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Foreword

Migratory fish have an outstanding swimming capacity. They travel widely across the oceans and visit different environments such as seawater, freshwater and the like. They have therefore all sorts of qualities unseen in other animals with regard to osmotic pressure adjustment, reproduction, behaviour and the like. Examining and explaining these characteristics is from the viewpoint of basic biology extremely useful to explain the phenomenon of life.

Meanwhile, as the present political and economic problems between countries regarding the question of the 200 nautical miles, of fishing restrictions in the North Pacific and regarding other subjects keep worsening, it becomes urgently necessary to increase artificially the marine resources on the evident basis of bioengineering for the purpose of fisheries.

Yet, few researchers are active in these fields of research. These fields are new and comprise countless untouched research topics. There is presently an increasing need to base the efforts on experimental biological studies considering thoroughly the various aspects of the fish migrations.

As a milestone for new developments of research in migratory fish, we will base ourselves on the recent study results and focus on the migratory phenomenon among fish. From the perspective of endocrinology, reproductive physiology, animal behavioral science and fisheries science,

we will present the latest views about molecular levels, cell - tissue - organ levels, as well as individual levels and phyletic formation levels. This book was compiled in the hope that it might spur the involvement of new people in these new research fields. If this happened, the authors and editors would have been made most happy.

The editors

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The present translation was produced by five different translators with the result that there are slight differences in style and possibly terminology (for instance "shirozake" may be translated as "chum salmon" in one chapter and as "dogsalmon" in another

The Biology of Migratory Fish

by

Masaaki MORISAWA, Katsumi AIDA,
Tetsuya HIRANO

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3. Peptide hormones of fish (26)

By Hiroshi KAWAUCHI

1. Foreword

Hormones are defined as chemical information transmitting substances which are produced in specific organs, get released into the blood and reach the target cell, and regulate complex physiological phenomena. With the recent increase in knowledge on peptide hormones and the structure of their genes, it became clear that there is a similarity in structure between hormones that differ in function. Furthermore, as microanalytic methods such as the radioimmunoassay method (RIA), immunological system staining method and high speed liquid chromatography became established and the sphere of research expanded, brain hormones were identified in the digestive tract and digestive tract hormones in the brain or central nervous system. Also, peptides resembling the hormones of mammals in structure were detected even in invertebrates and monads, and it became clear as well that hormones or molecules similar to them are distributed extremely widely.[1)] That is, peptide hormones may be said to be one group of information transmitting substance wherein peptide genes repeatedly duplicated and diversified in the process of biological evolution as an information transmitting signal to neighbouring cells. Consequently, in order to understand the chemical information transmission system formulated by such

a complex process, a perspective into the history of hormone matter, that is, molecular evolution and differentiation of function, is required. While the chemical information transmission system of fish produces peptide hormones more or less common in vertebrates, unique organs such as hypophysis caudalis and Stannius corpuscle also exist, which are extremely important objects of research in learning about the evolution of the information transmission system. From such a perspective, we have in this chapter put together the recently rapidly advancing knowledge on the hormones of fish and the structure of their genes.

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2. Hypothalamus hormones

2.1 Neuroderm hormones

Throughout vertebrates, the nucleus in the anterior part of the hypothalamus synthesizes hormone precursors which, while being transported to the neuroderm of the pituitary gland, become neuroderm hormones consisting of nine amino acid residues and carrier protein (neurophysine) consisting of approximately 90 residues. With respect to the former, except for Cyclostomata, in vertebrates across the board two structurally similar types of neuroderm hormones have been identified.[2] In mammals there are arginine vasopressin (AVP) which has a blood pressure elevating function and an antidiuretic function and oxytocin (OT) which has the function of contracting the uterine muscle and ejecting milk. As AVP series hormones, lysine vasopressin and phenypressin of marsupials, pig and hippopotamus have been identified as mutants. Outside of mammals, there is arginine vasotocin. As OT series hormones, mesotocin has been identified in lungfish to birds, isotocin in Teleostei, and glumytocin, barytocin or aspartocin in Elasmobranchii. The above-mentioned ten types of neuroderm hormones have an S-S bond in position 1 and position 6, and mutations can be seen in positions 2, 3, 4 and 8 (Fig. 1). With respect to the carrier protein, the structure of the two types of neurophysine and of the precursor mRNA in mammals has been determined, but the structure of neurophysine in fish is undetermined.

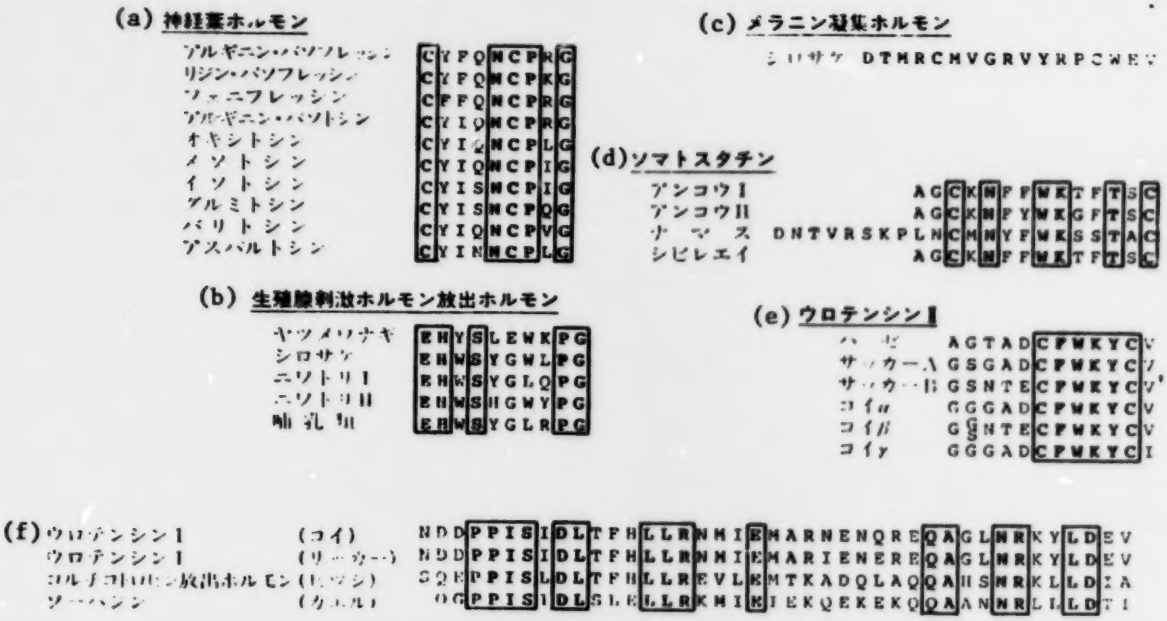

Fig. 1. Amino acid configuration of neurohormones.

(a) Neuroderm hormones
- Arginine vasopressin
- Lysine vasopressin
- Phenypressin
- Arginine vasotocin
- Oxytocin
- Mesotocin
- Isotocin
- Glumytocin
- Barytocin
- Aspartocin

(b) Gonadotrophic hormone releasing hormones
- Lamprey eel
- Chum salmon
- Chicken I
- Chicken II
- Mammals

(c) Melanin cohering hormones
- Chum salmon

(d) Somatostatin
- Angler I
- Angler II
- Catfish
- Electric ray

(e) Urotensin II
- Goby
- Sucker A
- Sucker B
- Carp α
- Carp β
- Carp γ

(f)
- Urotensin I (Carp)
- Urotensin I (Sucker)
- Corticotropin releasing hormone (Sheep)
- Sorbasine (Frog)

The similarity between the AVP series and OT series structure indicates that the two formed by a duplication of genes, but from the fact that there are only AVP series hormones in Cyclostomata, the duplication is surmised to have occurred in the early stage of the evolution of vertebrates. In Teleostei, arginine vasotocin is said to take part in diuresis and isotocin in copulation and spawning. The physiological function cannot be said to be certain. In this way, the molecular evolution of AVP/OT series peptides became clear, but there are many points that are unclear about their function in lower vertebrates.

2.2 Melanin cohering hormones

The body colour of lower vertebrates is regulated by the dispersion and cohesion of the melanin granules in the melanin cells. As the factor melanotropin (MSH) which promotes the dispersion of melanin, α-MSH and β-MSH are secreted from the pituitary intermediate lobe cells. On the one hand, the fact that a factor which promotes the cohesion of melanin is present in the pituitary gland of fish had been reported from the 1950s, but only recently was it isolated and structurally determined for the first time from the pituitary gland and brain of chum salmon.[3] This hormone is a new peptide which consists of 17 amino acid residues and has a S-S bond between position 5 and position 14 (Fig. 1). By (29) virtue of the fact that this peptide causes the melanin of Teleostei to cohere in vivo and in vitro, it is called a melanin cohering hormone (MCH). The fact that MCH is synthesized as a precursor in the nerve cell on the outer side of the outer apophysal nucleus of the hypothalamus and is one type of neurohormone which gets released into the blood from the neuroderm, was verified by the immunological system staining method and RIA. While peptides resembling MCH in other vertebrates are unidentified, a peptide which has both salmon MCH-like immunoactivity and melanin cohering activity for fish can be discerned in the hypothalamus of rat as well. Consequently, MCH-like peptides are, similar to AVP/OT group hormones, surmised to be present in vertebrates in common, with

their structural characteristics being preserved throughout evolution. However, the MCH of salmon functions in amphibians and reptiles in the same way as MSH. Consequently, its function is surmised to be diverse in the various classes of vertebrates.

2.3 Releasing hormones and inhibiting hormones

The secretion of pituitary hormones is regulated by releasing hormones and inhibiting hormones secreted from the hypothalamus. In mammals, thyroid-stimulating hormone releasing hormones (TRH), gonadotrophic hormone releasing hormones (LRRH, GnRH), somatostatin (ST) which inhibits the secretion of growth hormones, corticotropin releasing hormones (CRH) and growth hormone releasing hormones (GRH) have been identified.

ST is a typical brain-enteron hormone, but also exists in tetrahemena and colitis germs as well. In the case of ST of fish, two types of mutants have been isolated from the pancreas of angler, one of which has the same structure as ST of mammals (Fig. 1). The basic configuration of their precursor cDNA has also been determined.[4] The ST of catfish and electric ray has also been identified from the pancreas.[5,6] Catfish ST consists of 22 amino acid residues.

GnRH has been identified with respect to lamprey, salmon and chicken as well.[7] In every case it consists of ten residues similar to mammals, with the N-end being pyroglutamic acid and the C-end being amidated. Mutations occur in positions 3, 5, 6, 7, 8 (Fig. 1). In the case of GnRH, a similarity in structure to the bond promoting factor (α factor) of yeast has been pointed out.[8]

GRH and CRH have not been identified, but by virtue of the fact that the structure of urotensin I in the hypophysis caudalis is markedly similar to mammal CRH, the possibility for the two being the same substance is high. (30)

3. Hypophysis caudalis hormones

There are nerve secreting cells called hypophysis caudalis in the spinal tip of teleosts, which secrete urotensin I (U_I) and urotensin II (U_{II}).[9]

U_I is a factor which has both a blood pressure elevating function and a smooth muscle contracting function for rats. U_{II} is a factor which has both a blood pressure elevating function and a smooth muscle contracting function for fish. U_I of both sucker and carp consists of 41 residues, with mutations in two residues. U_I indicates a roughly 50% homogeny relative to the CRH of mammals and sorbasine isolated from the skin of frogs (Fig. 1). The basic configuration of cDNA, a carp U_I precursor protein, was also determined. As can be surmised from the similarity in structure, the three have in common an adrenocorticotrophic hormone (ACTH) secretion promoting function and a blood pressure lowering function. U_{II} of goby, carp and sucker has one S-S bond and consists of 12 residues. Mutants exist in carp and sucker. U_{II} and ST have a common Phe-Trp-Lys within the ring, but the size of the S-S ring differs (Fig. 1). As can be surmised from the similarity in structure, in the two can be seen a duplication of activity, such as inhibiting the secretion of prolactin (PRL) or the discharge of Cl ions.

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4. Pituitary hormones

4.1 Growth hormones and prolactin

Growth hormones (GH) manifest a growth promoting function in common among vertebrates. In addition to that, in fish they are known to raise their seawater adaptability as well. Prolactin (PRL), on the other hand, has extremely diverse functions for the various vertebrates, and in fish it manifests an osmotic pressure regulating function. Among fish, from salmon, eel, Tilapia, carp, sturgeon and shark various hormones have been refined, and the primary structure of salmon GH[10] and PRL,[11] eel GH,[12] carp PRL,[13] Tilapia PRL and the basic configuration of cDNA of salmon GH,[14] salmon PRL, eel and yellowtail GH have been determined as well. GH consists of approximately 190 amino acid residues, and has a large S-S ring in the centre of the molecule and a small S-S ring in the C-end. Fish PRL has two S-S rings in positions homologous to mammal PRL, but because it lacks a S-S ring at the N-end, the characteristics of the whole

(31)

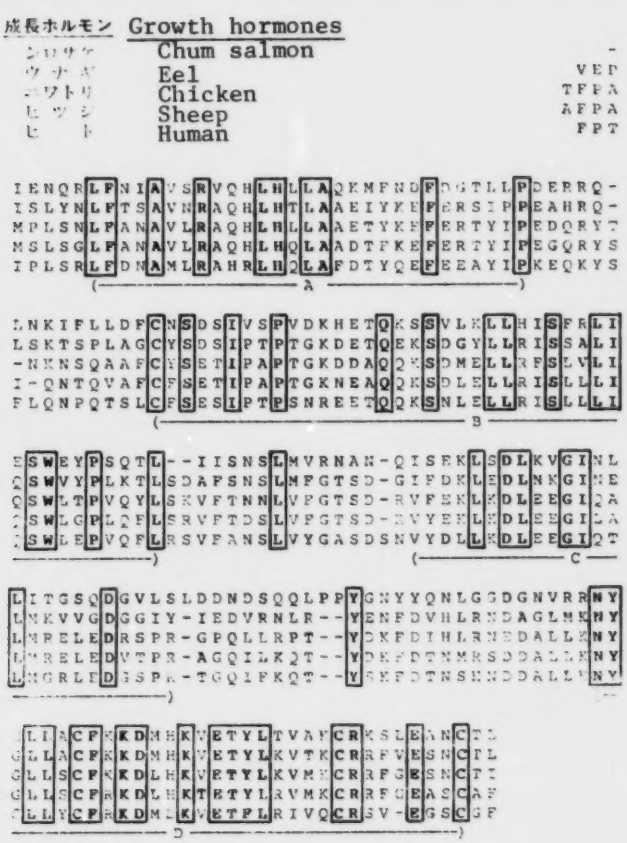

Fig. 2. Amino acid configuration of growth hormones.

molecule resemble GH. The homogeny of the amino acid configuration of salmon GH and PRL is no more than 26%. This is about the same as the similarity between the two hormones in mammals. GH and PRL, from their similarity in structure, have been surmised to be hormones produced by a duplication of common ancestral genes, but these results indicate that GH and PRL of fish are clearly differentiated not only in structure but in function as well. Surprisingly enough, eel GH has a higher similarity to chicken and mammal GH than salmon GH, which indicates that the hormones of fish, of which there are more than 20,000 current species, cannot be considered in the same way. (32)

On the other hand, mutants exist in GH and PRL of fish. This fact is worthy of special mention. Differences between the two types of mutants occur

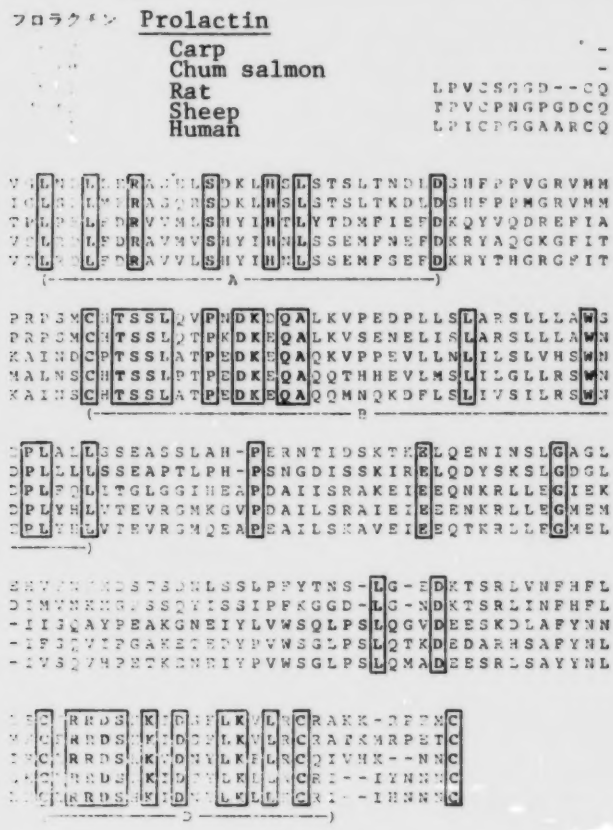

Fig. 3. Amino acid configuration of prolactin.

in 12 residues out of 188 in the case of salmon GH, four residues out of 187 in salmon PRL and one residue out of 187 in carp PRL. One of the mutants of eel GH consists of 188 residues similar to salmon GH, but the other is a molecule with three residues added to the N-end, with no other mutation being discernible. Tilapia secretes PRL of 20K and 24K in molecular weight. This difference in molecular weight is due to a deficiency of 5 - 6 residues each on the N-end side of the first and second Cys residue from the N-end, but because the two are markedly different in amino acid configuration as well, clearly they derive from two types of separate genes. In human GH as well exist mutants of 20K and 22K, but in this case it has been verified to be because two types of mRNA differing in chain length are produced at the time mature mRNA is produced from a mRNA precursor transferred from the same gene. (33)

Comparing the structure of GH of fish, chicken, sheep and humans, there are four segments (A, B, C, D) of high homogeny (Fig. 2). Among PRL the homogeny of the segments which correspond to segments A, B and D of GH is high, but the homogeny of segment C is no more than 2% (Fig. 3). Consequently, the amino acid configuration of segment C can be said to be specific to GH. In fact, the active fragment, positions 96 - 134, obtained by limited decomposition of trypsin in cow GH corresponds to segment C.

4.2 Pro-opiomelanocortin-related hormones

Pro-opiomelanocortin is synthesized in the ACTH cells of the anterior lobe and MSH cells of the intermediate lobe, and incurs zymolysis specific to the respective cells. From the anterior lobe are secreted mainly ACTH and β-lipotropin, while from the intermediate lobe are secreted α-MSH, β-MSH and β-endorphin (EP). In mammals the peptide which originates in the N-end of this precursor protein has a MSH-like structure (γ-MSH) and sugar chain. The structure of all peptides originating in α-, β-, γ-MSH and ACTH of shark and pro-opiomelanocortin of salmon has been determined[15] (Fig. 4). In salmon two types of mutants exist for all hormones, one of which is high in similarity to mammal hormones. In N-end peptide, the precursor of salmon, no γ-MSH-like structural segment nor sugar chain exists. Recently, the basic configuration of cDNA, the precursor hormone of salmon, has also been determined, and the structural characteristics of these has been verified at the gene level as well.[16]

α-MSH_I of salmon is consistent with mammal hormones. The N-end amino group has been acetylated and the C-end carboxyl group has been amidated. α-MSH_{II} of salmon consists of 15 residues, with the N-end being acetylated but the C-end being free. In the case of α-MSH of shark, in addition to position 13 being mutated into Met, the N-end and C-end are free. In mammals and shark they consist of 18 residues, but the two mutants of salmon both consist of 17 residues. β-MSH compared to α-MSH has greater mutation between species. Salmon β-EP_I and $_{II}$

(34)

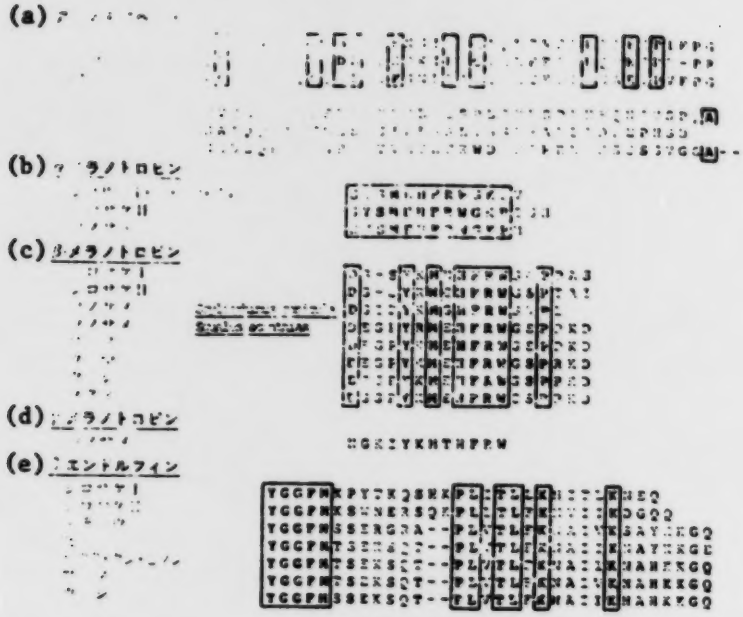

Fig. 4. Amino acid configuration of pro-opiomelanocortin-related hormones.

(a) Amino-end peptides
- Chum salmon I
- Chum salmon II
- Cow

(b) α-melanotropin
- Chum salmon I, ostrich, mammals
- Chum salmon II
- Dogfish

(c) β-melanotropin
- Chum salmon I
- Chum salmon II
- Dogfish — Scyliorhinus canicula
- Dogfish — Squalus acanthias
- Pig
- Horse
- Cow
- Camel

(d) γ-melanotropin
- Dogfish

(e) β-endorphin
- Chum salmon I
- Chum salmon II
- Ostrich
- Human
- Camel, cow, sheep
- Pig
- Cow

consist of 29 residues and 30 residues, respectively. β-EP with a free N-end amino group is extremely minute in quantity, the bulk of it being acetylated. Comparing the structure, the smallest active segment of MSH, His-Phe-Arg-Trp, and the smallest active segment of β-EP, Tyr-Gly-Gly-Phe-Met, are perfectly preserved throughout vertebrates.

4.3 Gonadotrophic hormones and thyroid-stimulating hormones

Gonadotrophic hormones (GTH) and thyroid-stimulating hormones are glycoproteins which consist of an α chain and β chain. The α chain is common among hormones, while the structural and functional characteristics of hormones derive from the β chain. From amphibians to primates, with some exceptions, two types of GTH, a luteinizing hormone and a follicle-stimulating hormone, have been identified. In fish, however, there was thought to be only one type of molecule resembling the GTH of mammals, and the gene duplication of GTH had been thought to have occurred in the process of evolution from fish to amphibians. However, the author et al.[17] isolated two types of GTH which take part in sex maturation (35) from salmon and carp. By virtue of the fact that both consist of α and β chains and that the β chain differs in amino acid configuration, they are molecules that belong to the erstwhile GTH. Consequently, the GTH genes can be said to be duplicated in Teleostei. On the other hand, Idler et al.,[18] using lectin affinity chromatography, fractionated salmon pituitary extracts and separated GTH of high and GTH of low sugar content, and have suggested that the former takes part in sex maturation while the latter is a new GTH which promotes the synthesis of yolk protein. Also, thyroid-stimulating hormones have been isolated from the pituitary gland of salmon, but there is no knowledge of the structure. In this way, there are still many unsolved questions about the type and function of glycoprotein hormones of fish.

5. Insulin

The role that insulin played in the development of endocrinology and

protein chemistry has been extremely big, and protein chemistry may even be said to have begun from the structural determination of insulin. Insulin manifests a marked blood glucose lowering function, and because of its high utility value as a treatment drug for diabetes, it is being isolated and refined from various vertebrates. In fish, the insulin of tuna, bonito, carp, angler, cod, toadfish, coho salmon, chum salmon, dogfish and hagfish has been structurally determined.[19,20] Insulin consists of an A chain and B chain, and in general the A chain consists of 21 residues and the B chain 30 residues, but the size of the B chain in fish is irregular. Within the A chain there is one S-S bond, and between chains there are two S-S bonds. While some mutations are discernible between species, the physicochemical properties of the corresponding amino acid residues are extremely similar (Fig. 5). It was in insulin that the identification of hormone precursors and the fact that active hormones get formed from precursors by being specifically enzyme-severed at the position where two or more basic amino acids are strung, were first made clear. In the precursor hormone, the B chain is positioned at the N-end and the A chain at the C-end, between which is a C chain. The structure of pro-insulin of angler, hagfish and salmon has also been determined. Insulin is high in structural similarity to insulin-like multiplicative factors, relaxin or nerve growth factors, while insulin-like substances are detected even in the digestive tract system, tetrahemena and colitis germs of invertebrates, and are hormones distributed most widely.

6. Calcitonin (36)

Calcitonin is a hormone which has a powerful serum Ca and P lowering function in mammals, but the function in fish has not been made clear. However, by virtue of the fact that the calcitonin of salmon and eel manifests in mammals an activity 30 - 50 times that of the mammal hormone, it is being utilized as a medical drug. The calcitonin of humans, cow, sheep, pig, rat, chicken, salmon and eel has been structurally determined.[21] In salmon there are three types of

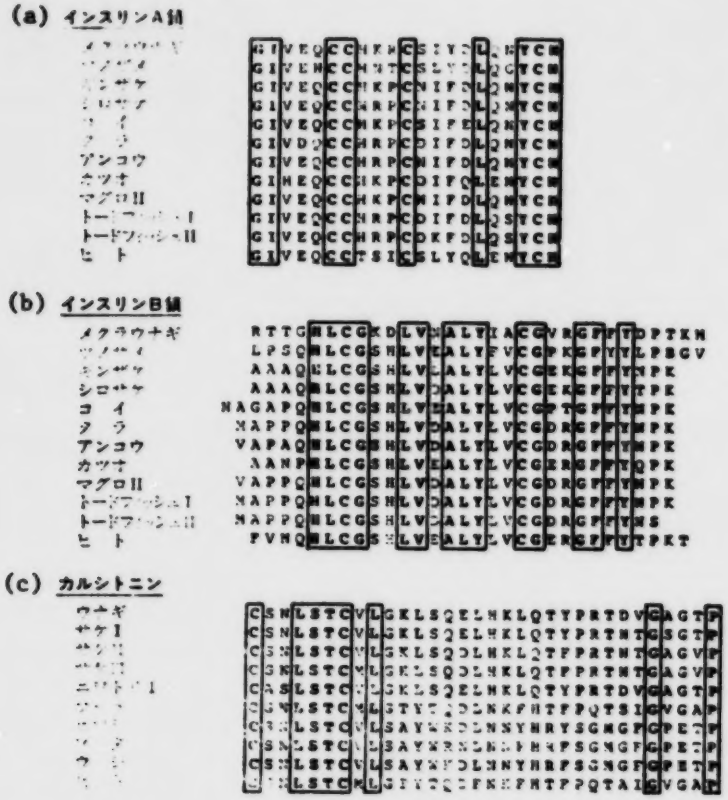

Fig. 5. Amino acid configuration of insulin and calcitonin.

(a) Insulin A chain
- Hagfish
- Dogfish
- Coho salmon
- Chum salmon
- Carp
- Cod
- Angler
- Bonito
- Tuna II
- Toadfish I
- Toadfish II
- Human

(b) Insulin B chain
- Hagfish
- Dogfish
- Coho salmon
- Chum salmon
- Carp
- Cod
- Angler
- Bonito
- Tuna II
- Toadfish I
- Toadfish II
- Human

(c) Calcitonin
- Eel
- Salmon I
- Salmon II
- Salmon III
- Chicken I
- Rat
- Sheep
- Pig
- Cow
- Human

mutants, and the structure of a total of ten types of calcitonin is known. All consist of 32 amino acid residues, have a S-S bond between position 1 and position 7, and the C-end carboxyl group is amidated. The non-variable residues are in positions 1, 4, 5, 6, 7, 9, 28 and 32 (Fig. 5). From the similarity in structure, it can be divided into a pig-cow-sheep group, human-rat group and chicken-salmon-eel group. This also corresponds well with the biological activity factor, the last group being the highest in acvitity. (37)

7. Conclusion

OT/AVP, GH/PRL, MSH/ACTH, GTH/TSH and insulin-group hormones are all hormones produced by the duplication of common ancestral genes. Consequently, it is clear that gene duplication is the mechanism of the evolution and functional differentiation of hormones. In other words, this gene duplication is not an extremely rare event that occurred just once in the early stage of the evolution of vertebrates, but may be thought to be something that occurs with a high frequency. For example, while it is unclear whether or not the two types of mutants of the GH-PRL group of hormones of fish all derive from separate genes, at least the two types of mutants seen in the GH and PRL of salmon and the PRL of 20K and 24K of Tilapia are ones that occurred as a result of the duplication of genes. In the GH-PRL group in mammals, on the other hand, placental lactogen (PL) and proliferin of mammals are known. From the fact that PL is high in similarity of structure to GH and proliferin to PRL, it had been thought that after GH and PRL had ramified, PL ramified from GH and proliferin from PRL. In point of fact, human GH and PL genes are on the same chromosome, but are positioned on a different chromosome to PRL genes. Furthermore, there are two human GH genes, one of which is a false gene. There are three PL genes, two of which express themselves while one is a false gene. In this way, as long as one of the duplicate genes maintains the inherent function, the other is thought to accumulate mutation and in many cases turn into a false gene and become inactive.

Then, at some opportunity or other a gene that acquires a new function is thought to emerge. This may be said to be the mechanism of evolution of a chemical information transmission system.

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CHAPTER 5: MIGRATIONS AND METABOLISM

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Yasuo INUI

1. CHARACTERISTICS OF FISH METABOLISM

It can be stated that the mechanisms that control the metabolic pathways and metabolisms of fishes are fundamentally not very different from those of the mammals and birds. However, with regard to the individual metabolic systems and the role of each metabolism in the metabolism as a whole, fishes differ from higher vertebrates in many aspects, and some differences are observed depending on the species of fishes. These differences often play a significant role in the way fishes live, including migration. In this chapter, the characteristics of fish metabolism as well as metabolic control are described first, centering on the salmonid fishes and eels.

1.1 Sugar Metabolism

It is a well known fact that fishes have a low capacity for the utilization of sugar and that they are dependant for

the most part on the protein and amino acid metabolisms. Phillips et al.[1] reported that when the carbohydrate content of the feed exceeds 12% the arrestation of growth and abnormal accumulation of glycogen take place with rainbow trout. Although it was reported that the capacity for the utilization of carbohydrates is increased considerably by the addition of an appropriate amount of vitamins and by improving the balance of the nutrients, in carnivorous fishes such as salmon, trout, eels and young yellowtail some form of disorder seems to manifest itself when the sugar content in the feed exceeds 40%, and, for this reason, it is correct to consider that the appropriate sugar content of the feed is at most 20%. When this amount is compared with 40 - 60% for mammals and birds, it can be understood how low the sugar requirement of fishes is. When the <u>in vivo</u> sugar turnover ratio was examined using labelled ^{14}C and the sugar utilization coefficient calculated from this value, it was found that in rainbow trout the turnover ratio was 360 minutes[2] and the coefficient of utilization was 4.5 μmol/hr for each 100 g of body weight[3] and in coho salmon the turnover ratio was 418 minutes and the utilization coefficient 11.7 μmol/hr for each 100 g of body weight[4], these values being only 1/10 of those found in rats. In this manner, the utilization of sugar in fish is low but when the carbohydrate content of its food in its natural environment is considered, it seems safe to state that it is necessarily so.

1.2 Protein Metabolism

In contrast to the case of carbohydrates, the requirements for proteins or amino acids in fishes far exceeds those in rats and chicken - said to be more than double at times. In mammals, the energy contribution by proteins is extremely small in comparison to the energy produced by carbohydrates and fats under normal conditions, whereas in fishes the energy production as a result of the catabolism of proteins normally accounts for 40% or more of the total energy produced, at times reaching as much as 85%[5]. Proteins are not only involved in energy production but are also extensively utilized as the substrate for the neogenesis of sugar and the synthesis of fats. Although in mammals too, with the exception of the ruminants, sugar is generated for the most part from amino acids, since the synthesis of fats utilizes mostly sugar, the ingestion of a large amount of sugar results in the accumulation of fats. On the other hand, in fishes amino acids are mainly used as the substrate for the synthesis of fats, as will be explained later.

The synthesis of proteins from amino acids ingested into the body or the catabolism of proteins to amino acids in fishes is thought to differ very little from that in higher vertebrates. It can be stated that in nitrogen metabolism the characteristics specific to the living organisms are most strongly manifested in the end products as well as the form in which they are discharged. All animals produce ammonia as the

metabolite of proteins. However, because ammonia is highly toxic it is excreted after being changed into the form of urea or uric acid depending on the habitat, with the exception of aquatic organisms. However, in the case of fishes, 50 to 80%[6)&7)] of the total nitrogen excretions in carp and 50 to 60%[8)] of the total nitrogen excretions in rainbow trout are excreted as ammonia and the percentage for other ocean fishes are 50 to 90%[9)]. It can be stated that the ability to discharge the end product of the proteins out of the body directly in the form of ammonia without requiring the energy to convert it into other substances in this manner is highly advantageous to fishes, in which the protein metabolism plays a major role.

It cannot be said with certainty that the mechanism for the formation of ammonia from amino acid in fishes is fully understood. The manner in which ammonia is formed from amino acids in animals can be roughly divided into two categories. The first is the formation of ammonia directly from amino acids by the action of specific enzymes and the other is the deamination of the amino radical of the amino acid via a common acceptor, and it is thought that the latter process plays a larger role in fish. Two processes given in Figure 1 are known for this indirect metabolic system[5)&10)]. One is a system called the amino-group transfer reaction in which α-keto acid and ammonia are formed from α-amino acid by the action of aminotransferase and glutamic acid. The other is a process

called purine nucleotide cycling and in this case α-amino acid becomes aspartic acid by the actions of two kinds of aminotransferase and enters the purine nucleotide cycle to produce ultimately α-keto acid and ammonia. It is thought that in rainbow trout the process by the aminotransfer reaction plays a much larger part than the one of purine nucleotide cycling[10]. Then where does the formation of ammonia by deamination take place? The finding that the amino acid content in plasma is markedly increased when the liver of eel is extracted (Figure 2)[11] suggests that the liver is the main organ where deamination takes place. On the basis of findings demonstrating that the amount of ammonia excreted remained unchanged in eels whose liver was removed, Kenyon reported that the liver does not have a great involvement in deamination in eels[12], however, this leaves no explanation for the increase in the amino acid content in plasma at the time the liver is extracted. There is a strong possibility that a compensation of some kind for ammonia excretion will be in force under such abnormal physiological conditions as the removal of the liver. After determining the ammonia content of blood in various kinds of blood vessels in carp, Pequin and Serfaty[13] concluded that the liver is the principal organ involved in the formation of ammonia and that the kidneys and muscles also participate in the production of ammonia. On the basis of the findings of these studies as well as on the distribution state of enzymes, it is thought that in fishes the liver is normally, and at the

time of feeding, in particular, the principal organ responsible for the formation of ammonia and that the kidneys and muscles also participate in deamination during fasting[10].

The movements of proteins and amino acids in fish described up to this point were consolidated and shown in Figure 3. As demonstrated in this diagram, the amino acids contained in the blood are supplied by the amino acids formed by the decomposition of proteins and amino acids contained in food and body proteins, and they are utilized in the synthesis of proteins in various parts of the body and mostly deaminated in the liver.

When the amino acid content in the blood of eels was examined it was found that it remained fairly stable at 4 - 9 mg/dl normally, though it increased immediately after the ingestion of food, and this indicates that a control mechanism to maintain the amino acid content in the blood at a constant level is in force. Although the administration of insulin to eels results in decreased blood sugar, as in mammals, it also results in almost all of the plasma amino acids to decrease at the same time (Figure 4)[14]. In contrast to this, the administration of alloxan, which is known for its ability to inhibit the β-cells of the islets of Langerhans, results in a rise in most of the amino acids (Figure 5). When the islets of Langerhans are removed from American eels, too, the plasma amino-form nitrogen shows an increase temporarily[15]. When the plasma amino acids of eels administered with alloxan were

examined, it was found that although essential amino acids were greatly increased the increase in nonessential amino acids was relatively small (Figure 5). However, when alloxan was administered to eels whose liver had been removed, it was found that nonessential amino acids were also markedly increased, with the exception of special amino acids such as taolin*[14]. These findings indicate that, although the administration of alloxan, i.e. a shortage of insulin, causes in actuality almost all of the amino acids to be mobilized, because nonessential amino acids are deaminated with a relative swiftness in the liver they do not appear to a great extent as an increase in 59
the blood. The plasma amino acids of eels can be roughly divided into three groups according to their reaction with regard to the extraction of liver and the administration of insulin and alloxan. Those that belong to the first group are the amino acids that are mobilized by the shortage of insulin but have a relatively slow rate of metabolism in the liver and they include essential amino acids such as threonine, valine, methionine, isoleucine, leucine, phenylalanine, and arginine as well as tyrosine and proline. The amino acids belonging to the second group are mobilized by the shortage of insulin and are metabolized in the liver quickly and they include most of nonessential amino acids such as aspartic acid, serine, asparagine or glutamine, glutamic acid, glycine, alanine, ornithine and essential amino acids such as ricin and

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* Translator's note: probable spelling.

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histidine. Those belonging to the last group include taolin, methionine sulfoxide, citrulline and α-amino-n-butyric acid and these differ from the ones belonging to the first two groups in that they do not increase even when the liver is removed. In other words, they are not mobilized internally and thus it can be said that they have a different significance from the normal amino acid metabolism in the body[14].

Seshadri[16] reported that when insulin is administered to murrell (Ophicephalus striatus) the free amino acids in muscles were decreased and the amino acids that are bonded to proteins were increased. In toadfish[17] and northern pike[18], insulin facilitates the synthesis of proteins from radioactively labelled amino acids (^{14}C-glycine and ^{14}C-leucine) in the muscle. Insulin also facilitates *in vitro* the synthesis of proteins from ^{14}C-glycine in the liver and branchial mantle muscle in eels[19]. Although α-aminoisobutyric acid is incorporated into the cell in a similar manner to other amino acids, it remains in the cell in that state since no system exists to metabolize it after it is incorporated. The labelling of this amino acid with radioactivity enables one to examine the amino acid uptake rate of cells apart from the synthesis rate of proteins. Although insulin facilitates the uptake of ^{14}C-glycine into proteins in the liver, the radioactivity of the fraction (mostly amino acids) soluble in trichloroacetic acid (TCA) is rather reduced. However, when α-aminoisobutyric acid is used, it becomes clear that the uptake

of amino acids into the cells is also increased. In the case of glycine, the results indicate that the action of the insulin to facilitate the protein synthesis exceeded the action to facilitate the uptake. On the other hand, even in the case of glycine, the radioactivity in the TCA fraction as well as in proteins are increased in the muscle (Table 1)[19)&20)]. These differences are thought to be caused by the differences in the rate of protein synthesis in internal organs. As demonstrated here, insulin has the dual effect of facilitating the uptake of amino acids into cells as well as facilitating the protein synthesis in both the liver and muscle tissues.

The injection of glucose into the blood of eels causes the insulin content in the blood to rise, but the administration of such amino acids as arginine and ricin also causes the facilitation of the insulin secretion to a similar or greater level. The simultaneous administration of arginine and glucose causes the insulin secretion to continue for a longer period of time than the administration of these substances alone[21)]. The facilitation of the insulin secretion from the islets of Langerhans by glucose and leucine in toadfish was confirmed in <u>in vitro</u> experiments also. In this instance, too, the reaction was greater when glucose and leucine were administered simultaneously than by itself[22)]. These facts demonstrate that the intake of food by fishes causes the blood glucose and amino acid levels to rise and facilitates the secretion of insulin and these in turn cause

the uptake of the amino acids in the blood into the tissues as well as the protein synthesis to be facilitated. In contrast to this, it has been observed that in fasting eels a decrease in the functions of the ß-cells of the islets of Langerhans takes place and a temporary increase in the plasma amino-form nitrogen level takes place during this time[23]. In this manner, insulin plays a major role in regulating the protein and amino acid metabolisms in fish.

The removal of the pituitary gland causes the cessation of growth in fishes and the growth is recovered by the administration of a growth hormone. In this case, it is known that this effect to facilitate the growth is not only limited to the fish growth hormone but a similar effect can also be obtained by the use of mammalian growth hormones. Detailed review on the action of growth hormone to facilitate growth in fishes has been conducted by Donaldson et al.[24]. In comparison to extensive research conducted on the growth facilitating action of growth hormones, there are only few instances of research that comment on the protein metabolism 61
controlling mechanism of the growth hormones in fishes. Cheema and Matty[25] reported that the protein synthesis was increased by administering porcine growth hormone to rainbow trout and examining the activity of radioactively labelled leucine in the muscle. In a similar manner, the administration of ovine growth hormone to eels facilitates the protein synthesis from leucine in the liver and muscle. However, in the case of eels,

unlike mammals, a considerable time is required for the protein synthesis action to be manifested as a result of the administration of the hormones[26]. Whereas in mammals this action manifests itself in a short time (within 30 minutes) even in *in vitro* situations, in the case of eels the protein synthesis facilitative effect of the growth hormones is not observed in either liver slices or branchial mantle muscle even with cultivation lasting at least 5 to 10 hours. The fact that the *in vitro* protein synthesis is increased when a liver slice prepared from an eel which had been administered a growth hormone previously (two days before)[26] leads one to wonder if some kind of *in situ* factors are necessary for the action of growth hormones to facilitate the protein synthesis to manifest itself.

The removal of the pituitary gland of an eel causes the amino-form nitrogen in the plasma to be decreased. The administration of mammalian growth hormone to this eel, in contrast, causes the amino-form nitrogen in the plasma to be increased. However, a considerably long period of time (two days) is required for this reaction to take place. This effect of the mammalian growth hormone to increase the amino-form nitrogen in plasma is also observed in eels whose pituitary gland has not been extracted (Figure 6)[27]. However, the fact 62
that this effect is not only limited to the growth hormone, since the administration of mammalian prolactine also produces a similar increase, necessitates a detailed investigation using

the hormones obtained from the same species in order to determine whether this increase in the amino-form nitrogen in the plasma is due to the action of growth hormones proper.

It is known that in humans and live stock animals, various sex hormones, synthetic androgen in particular, cause strong protein anabolism or the effect of muscle enhancement to take place. In fishes, too, the effect of weight gain caused by various androgen, estrogen, progestogen and synthetic steroids has been confirmed in some species, mainly in salmonid fishes[24]. However, relatively few studies have been conducted on the direct investigation of the facilitative action of these hormones on the synthesis of protein: the only reports being the one stating that dimethazine and norethandrolone facilitate the synthesis of muscle protein from ^{14}C-leucine in rainbow trout[24] and another report demonstrating that estradiol facilitates the synthesis of protein from ^{14}C-serine and ^{14}C-glycine in <u>in vitro</u> free cells of coho salmon[28]. On the other hand, Andō et al.[29] confirmed that the administration of 17 α-methyltestosterone to white salmon fry facilitated the protein catabolism actions, such as a decrease in the soluble protein in the muscle and an increase in the extract-form nitrogen. However, this hormone is a synthetic androgen that causes a very strongly felt effect of weight gain in fishes including salmonid fishes. The dosage (30 mg/kg in feed) used by Andō et al. is somewhat higher than the dosages (1 to 10 mg/kg) used in past studies in which

weight gain was observed by the use of hormones in salmonid fishes, moreover, there have been other instances in which very high dosages (50 to 100 mg/kg) have caused a weight loss[24]. Steroid hormones are pleiotropic hormones and their actions sometimes vary greatly depending on the dosage. Furthermore, since these hormones are known as the hormones that aid other hormones by providing them with an environment favorable to them, in other words hormones with strong permissible effect, there is a strong possibility that they act to augment the metabolisms of various kinds, depending on situations such as increase in the enzyme activity, instead of promoting unidirectional metabolism at all times. Inui et al.[30] observed that the administration of cortisol to eels causes an increase in the activities of enzymes that work in completely opposite directions, hexose diphosphatase and phosphofructokinase. Detailed explanation on the action mechanism of steroid hormones on protein metabolism in fishes is desired at this point.

Tyroid hormones are also considered to participate in some form in the protein metabolism in fish. The fact that the 63
administration of thyroxine (T_4)to goldfish causes an increase in the amount of nitrogen excreted at least under some conditions has been investigated by a number of research workers[31)&32)]. However, in contrast to this, it was stated by Smith and Thorpe[33] that the administration of T_4 to rainbow trout resulted in a decrease in the amount of nitrogen

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excreted. It was reported that the synthesis of protein from ^{14}C-leucine is facilitated by T_4 in the liver and gills of rainbow and brown trout in the case of Narayansingh and Eales[34] and by triiodothyronine (T_3) in the muscle of killifish in the case of Jachim and Laroche[35]. These actions of T_4 and T_3 are not only confirmed in in vivo situations but also in in vitro situations as demonstrated by the following observations: the synthesis of protein from leucine in the muscle of goldfish is facilitated by T_4[32] and the synthesis of protein from serine in the free cell of the liver of coho salmon is facilitated by T_3[28]. As demonstrated here, although the results of studies on the protein metabolism involving thyroid hormones in fish conducted by various investigators are not necessarily consistent, it seems that many of the investigators are in agreement on the point that a low concentration of thyroid hormones direct the amino acids towards the synthesis of protein[24].

1.3 Lipid Metabolism

Since several excellent outlines are in existence[36]-[38] on the subject of the absorption and transport mechanisms of fat in fish or on the metabolism of amino acids in fish, they should be consulted. In this article, only simple explanations concerning a few physiological characteristics of fat metabolism in fish as well as the control of internal secretions will be attempted.

It is known that, in mammals, besides the fats ingested in that form, many fats are synthesized from carbohydrates. However, in the case of fish, the carbohydrate content of naturally occurring food is low, and, therefore, the synthesis of fat from carbohydrate has no great significance. Nagai and Ikeda[39] determined that the uptake of glutamic acid by the neutral fats in the liver is far greater than that of glucose after injecting carp with radioactively labelled glucose or glutamic acid. As a result of investigations conducted on the uptake of radioactively labelled glucose, alanine and palmitic acid into the fats of the <u>in vitro</u> liver and adipose tissues in rainbow trout, Henderson and Sargent[40] reported that alanine and palmitic acid are incorporated into the fats of the liver slice more than glucose. On the other hand, it was found that palmitic acid was incorporated into the adipose tissue in a great quantity. Although some radioactivity was incorporated from glucose, the majority consisted of those that had entered the glycerol fraction of the fat. On the basis of these findings, it is thought that in rainbow trout the liver plays a central role in the synthesis of fat and that the adipose tissue is simply an organ for storing fat. In coho salmon, the fact that the enzyme activity of the fat synthesis system is high in the liver and low in the adipose tissue[41] has been revealed and this corroborates the above described findings. In this manner, the

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amino acid has a great significance in fish, unlike in mammals, as the substrate for the synthesis of fat.

Fat is utilized by the liver and many other organs as a source of energy and it is known that the metabolism in this instance, as in the case of mammals, is by what is called beta oxidation[10]. In mammals, as during the time of fasting, lipolysis becomes more rampant and when the amount of acetyl-CoA produced by the beta oxidation exceeds the amount that can be processed by the citric acid cycle (TCA cycle) the excess acetyl-CoA is directed towards ketone metabolism, thus resulting in the formation of a large quantity of ketone bodies. However, even when rainbow trout are placed in the state of fasting or even during the natural state of long-term fasting, as seen in sockeye salmon during the spawning migration time, the ketone body content in the blood does not rise[42]. Zammit and Newsholme[43] reported that the activity of 3-hydroxybutyric dehydrogenase was not detected in ocean dwelling teleost and that even when they are feeding or in the state of fasting no 3-hydroxybutyric acid, which is the most important ketone body in the blood of mammals, is detected in the blood of these fishes. In this manner, it is thought that, unlike in mammals, no formation of ketone bodies takes place in teleost even during the time of fasting[10].

It is known that in mammals catecholamine promotes the mobilization of fat, thus raising the amount of free fatty acid in the blood. This is due to the activation of adenylate

cyclase by catecholamine, thus promoting the production of cyclic AMP from ATP and ultimately activating lipase. Consequently, this action is not limited only to catecholamines but substances that activate adenylate cyclase such as adrenocorticotropic hormones (ACTH) and glucagon as well as cyclic AMP also have a similar effect and they have an important significance as the system mobilizing fat during emergency requirements, such as during times of stress. Farkas[44] discovered that in both *in vivo* and *in vitro* carp none of the epinephrine, norepinephrine or ACTH has the ability to mobilize fat. A rise in the free fatty acid is not observed even when epinephrine or glucagon is administered to the *in vitro* adipose tissue of tilapia (Inui, not yet published). On the basis of these findings, it is thought that no hormone-responsive lipase, which correspond to that found in mammals, is present in fishes. The fact that the no mobilization of fat by these hormones takes place in snakes or frogs either led Farkas to conclude that no hormone-responsive lipase is present not only in fishes but also in animals that are lower than the reptiles[45]. 65

It is known that thyroid hormones also have the action of mobilizing fat in mammals. In the case of fishes, too, there have been reports stating that the administration of thyroid hormones has the effect of lowering the fat in the liver and adipose tissues[46][47] and raising the free fatty acids in the blood[48]. On the basis of the results of surveys

conducted on various studies concerning the role of the thyroid gland on lipid metabolism in fishes, Plisetzkaya et al.[49] concluded that thyroid hormones take a part in the mobilization of fat. However, the administration of thyroid hormones in some cases yields results which deny that the mobilization of fat is taking place or reveals that a reaction which is totally opposite to the fat mobilization is taking place. Furthermore, it cannot be stated that the inhibition of the thyroid gland by radioactive rays or the administration of antithyroid substances such as thiourea or propylthiouracil always bring about a consistent effect on lipid metabolism[49].

The administration of mammalian growth hormones in rainbow trout and sockeye salmon causes the fat content of the muscle and body to decrease[24)&50]. In addition, the growth hormones cause an increase in the free fatty acid content of the blood in rainbow trout and eels[27)&51]. Naturally, there are some exceptions, but it is thought that, of the hormones that are involved in the mobilization of fat in fishes, the growth hormones are the ones having the most reproducible effects. In recent years, the fact that the growth hormones have the effect of accelerating the growth into smolts of salmon is becoming clear[52)-55]. It is a well known fact that the fat content of the body is decreased during the time of transformation from parr to smolt[53),56)&57]. Thyroid hormones are also involved in the change to smolts of salmon, and since there is a possibility that thyroid hormones are also involved

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in the mobilization of fat, as described before, the possibility that the thyroid hormones are involved in the lowering of the fat level that accompany this change cannot be denied. However, when only thyroid hormones are administered to parr before it turns to smolt, the storing of fat often takes place instead[55),56)], indicating that the possibility of the growth hormones playing a major role in the mobilization of fat accompanying the transformation from parr to smolt is much higher. In mammals, also, the mechanism of the mobilization of fat by the growth hormones is not necessarily clear. However, instead of directly activating the hormone-responsive lipase as in the case of catecholamine and glucagon, it is thought that the growth hormones act in the presence of factors that activate the mobilization of fat[59)]. As explained before, the fact that, in spite of the lack of hormone-responsive lipase in fishes, the possibility for the presence of the fat mobilization action of the growth hormones is thought to be of great interest from the standpoint of clarifying this action mechanism.

2. METABOLISM AT SPAWNING MIGRATION TIME

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The salmon that have ascended the river during spawning migration normally do not feed. However, not only do they consume a great deal of energy in travelling a long distance against the current, but also catabolism and anabolism that are specific to the development of the gonads are taking place during this period. Chang and Idler[60)] reported that the liver

glycogen in male rainbow trout was increased from 18 to 31 mg/g during spawning migration. In other words, this indicates that the neogenesis of sugar exceeds its utilization during this period, resulting in the storing of excess sugar as glycogen. A similar phenomenon is observed in eels, which have a prolonged period of migration for spawning, when they are made to fast. The fasting causes a rapid decrease in the liver glycogen in eels but once reduced it starts to increase gradually after that. Judging from the fact that the activity of transaminase in the liver as well as the amino-form nitrogen level in the plasma is increased during this time, it is thought that the neogenesis of sugar takes place from the amino acids produced by the decomposition of the body protein. Moreover, since a reduction in the beta cell function of the islets of Langerhans during this period was also detected, the mobilization of amino acids as a result of a reduction in the insulin level is suggested[61]. The fact that the catabolism of protein is also taking place in the case of salmon, as will be explained later, leads one to assume that the neogenesis of sugar during spawning migration takes place from amino acids. It is thought that in salmon the newly formed sugar, as well as stored sugar, is utilized in this manner during the emergency of spawning migration[62].

It is thought that sockeye salmon that swim up the Fraser River for a distance of over 1,000 km during spawning migration normally use fat or protein for the source of energy

during this time. It is known that although fat is the main source of energy during the first part of the migration protein becomes the metabolic substrate during the latter part of the migration[62)&63)]. After conducting a sampling of sockeye salmon that swim up the Fraser River at 300 to 400 km intervals, Mommosen et al. stated that the normal muscle is the main source of amino acid supply during spawning migration, on the basis of findings demonstrating that the soluble and insoluble protein fractions in the normal muscle are decreased by as much as 70% during this time while the protease is dramatically increased. Furthermore, on the basis of findings demonstrating that the alanine content of the amino acid pool in the muscle is higher than the theoretical value obtained from the proteolysis and that the levels of GPT (glutamic-pyruvic transaminase) and malic enzyme both of which are required in the conversion of amino acid are maintained high during spawning migration, they have concluded that the amino acid produced by the decomposition of normal muscle is converted to alanine and distributed through blood to be utilized by various parts of the body. Similar results have been also observed with dog salmon during spawning migration. During this period, the metabolic situation of dog salmon differs somewhat whether they are at sea or have migrated up the river. Although they are feeding while migrating in the ocean, the fat level, the triglyceride level in particular, is decreased during this time[29)]. This trend is especially more

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pronounced in females. Since this decrease in fat takes place side by side with an increase in the GSI and at the time when the levels of estradiol-17 β and testosterone are increased, Andō et al.[29] consider that this phenomenon plays a large part in the development of the gonads. On the other hand, the salmon stop feeding when they enter the river. During this time an increase in their protease activity is seen, as well as an increase in the autolysis of the muscles, a decrease in the level of soluble protein and an increase in the extract-form nitrogen and all of these changes led to the conclusion that the body protein is utilized as the source of energy required in climbing the river[64][65].

It is known that the blood corticoid is increased by six fold of the normal level in salmon during spawning migration[63]. It has been thought for a long time that this hyperadrenocorticism in salmon during spawning migration facilitates the catabolism of the muscles as well as the transfer of the substances to the gonads in the body[66]. On the basis of findings showing that the administration of 17 α-methyltestosterone to white salmon facilitates protein metabolism in a manner similar to that observed in salmon during the ascent of a river, as manifested by a decrease in the muscle protein, an increase in the extract-form nitrogen and an increase in the autolysis of the muscle, Ando et al.[67] stated that androgen is contributing to the proteolysis after they enter the river. It is thought that during this period

the raised levels of sex steroids and corticoids facilitate the catabolism and develope the gonads in a synergistic manner.

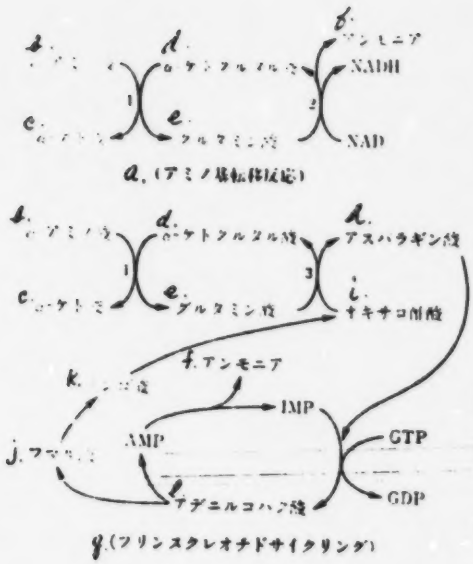

図1 アミノ酸からのアンモニア生成経路.
1:アミノトランスフェラーゼ
2:グルタミン酸デヒドロゲナーゼ
3:アスパラギン酸アミノトランスフェラーゼ

Figure 1: The process of ammonia formation from amino acids.
1: aminotransferase
2: glutamic dehydrogenase
3: aspartic aminotransferase

a. (aminotransfer reaction)	b. α-amino acid
c. α-keto acid	d. α-ketoglutaric acid
e. glutamic acid	f. ammonia
g. (purine nucleotide cycling)	h. aspartic acid
i. oxalacetic acid	j. fumaric acid
k. malic acid	l. adenylsuccinic acid

図 2 肝臓を摘出したウナギの血漿アミノ酸. ■ 肝臓摘出, □ 無処理

Figure 2: Plasma amino acids in hepatectomized eel.
■ hepatectomized □ untreated

1. threonine
2. valine
3. methionine
4. isoleucine
5. leucine
6. phenylalanine
7. ricin
8. histidine
9. arginine
10. taolin
11. methionine sulfoxide
12. aspartic acid
13. serine
14. asparagine glutamine
15. proline
16. glutamic acid
17. citrulline
18. glycine
19. alanine
20. α-amino-n-butyric acid
21. tyrosine
22. ornithine

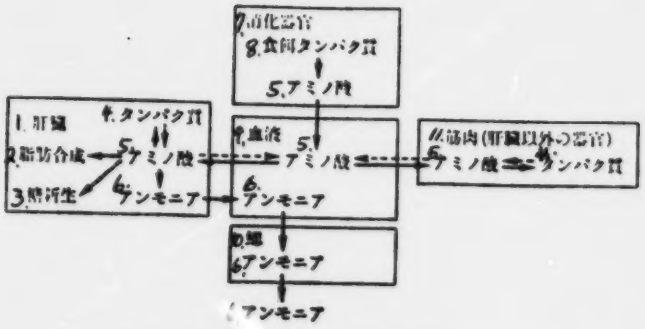

図3 魚類におけるアミノ態窒素の流れ

Figure 3: The flow of amino-form nitrogen in fishes.

1. liver
2. synthesis of fat
3. neogenesis of sugar
4. protein
5. amino acid
6. ammonia
7. digestive organ
8. food protein
9. blood
10. gills
11. muscles (organs other than the liver)

表1 ウナギ肝スライスおよび筋肉における ^{14}C-グリシン, ^{14}C-α-アミノイソ酪酸から TCA 可溶性分画ならびにタンパク質への放射能のとり込みに対するインスリンの作用

		1. グリシン		2. α-アミノイソ酪酸
		TCA*	3. タンパク質**	TCA*
4. 肝臓	5. 対照	459±33	2,814±357	152 ±18
	6. インスリン	339±31[a]	4,602±360[a]	201 ±23[b]
7. 筋肉	5. 対照	455±37	149± 13	92.5± 5.1
	6. インスリン	508±33[b]	220± 14[b]	99.0± 6.8[b]

8. インスリン 0.1IU/ml, * dpm/mg 組織, ** dpm/mg タンパク質.
9. a: $p<0.001$, b: $p<0.01$, c: $p<0.02$. ただし有意差の検定はそれぞれに対応する組織片の値を用いた pairing design test によっている.

Table 1: The action of insulin on the uptake of radioactivity from ^{14}-glycine, ^{14}C-α-aminoisobutyric acid into the TCA soluble faction and protein in the liver slice and muscle in eels.

1. glycine
2. α-aminoisobutyric acid
3. protein
4. liver
5. control
6. insulin
7. muscle
8. insulin 0.1 IU/ml, * dpm/mg tissue, ** dpm/mg protein.
9. a:$p<0.001$, b:$p<0.01$, c:$p<0.02$, however, the significance test was conducted by the pairing design test using the corresponding tissue slices.

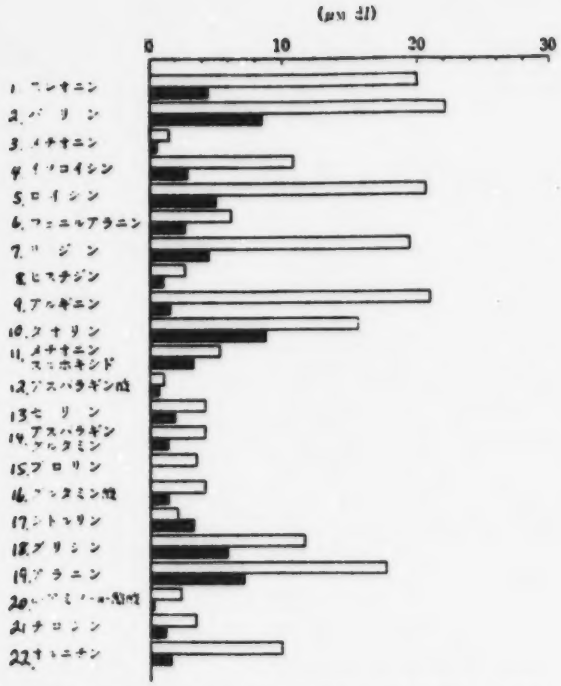

図 4 インスリン投与 (5IU/kg) がウナギの血漿アミノ酸に及ぼす影響. ■ インスリン投与, □ 生理食塩水投与

Figure 4: The effect of the administration of insulin (5 UI/kg) on the plasma amino acid in eels.
■insulin administered
□physiological saline solution administered

1. threonine
2. valine
3. methionine
4. isoleucine
5. leucine
6. phenylalanine
7. ricin
8. histidine
9. arginine
10. taolin
11. methionine sulfoxide
12. aspartic acid
13. serine
14. asparagine glutamine
15. proline
16. glutamic acid
17. citrulline
18. glycine
19. alanine
20. α-amino-n-butyric acid
21. tyrosine
22. ornithine

図5 アロキサン投与（250mg/kg）がウナギの血漿アミノ酸に及ぼす影響.
■ アロキサン投与,
□ 生理食塩水投与

Figure 5: The effect of the administration of alloxan (250 mg/kg) on the plasma amino acid in eels.
■alloxan administered
□physiological saline solution administered

1. threonine
2. valine
3. methionine
4. isoleucine
5. leucine
6. phenylalanine
7. ricin
8. histidine
9. arginine
10. taolin
11. methionine sulfoxide
12. aspartic acid
13. serine
14. asparagine glutamine
15. proline
16. glutamic acid
17. citrulline
18. glycine
19. alanine
20. α-amino-n-butyric acid
21. tyrosine
22. ornithine

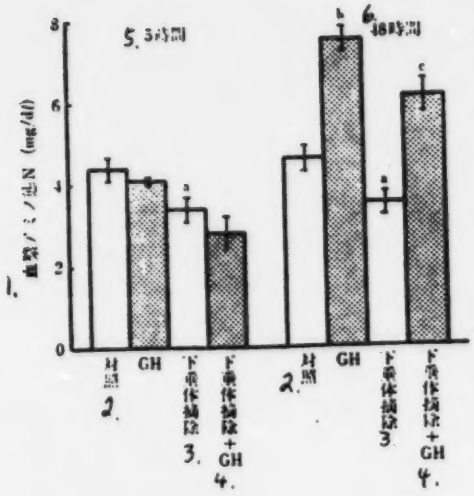

図 6 ウナギの血漿アミノ態窒素に対する成長ホルモン投与 (2 μg/g 体重) の影響.
a：対照に対して $p<0.05$
b：対照に対して $p<0.001$
c：下垂体摘除に対して $p<0.001$

Figure 6: The effect of the administration of growth hormones (2 μg/g body weight) on the plasma amino-form nitrogen in eels.
a: $p<0.05$ to the control
b: $p<0.001$ to the control
c: $p<0.001$ to the samples with the pituitary glands removed.
1. plasma amino-form N (mg/dl)
2. control
3. pituitary glands removed
4. pituitary glands removed + GH
5. 5 hours
6. 48 hours

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3.4 Salmonids

Many species of salmonid fish, which are divided into the genera of salmon, rainbow trout, char and hutcho trout show a life cycle of spawning in rivers -- hatching -- catadromous migration of smolt -- oceanic migration -- anadromous migration for spawning[1,10]. However, the period of freshwater or oceanic living varies with species: a few species had been landlocked in the cool-water region of upper river streams as a result of the warming-up after the ice age; in some groups smolt cannot descend the rivers to the sea because of dam construction and/or embanking, whereas in others prematurely developed individuals in rivers spend their entire life in freshwater areas. Thus salmonids represent a group of fishes that are of extreme interest in the examination of mechanism(s) responsible for migration. p. 80

3.4.1 Sexual maturation and catadromous migration

Salmonids in general develop faster with increasing length of daylight and spawn from fall toward winter. Eggs laid on the spawning ground of the river hatch in 60 to 70 days, depending on the species of fish and the condition of water temperature, several tens of days later the yolk is absorbed to develop into fry that are capable of free swimming. Fry that live in rivers are called parr, which are characterized by dark body color, the presence of plural numbers of oblong and black spots, called parr marks, on the sides of the body, and a high degree of fatness. The parr orient themselves in relation to the river current to establish their own feeding territories. The parr stage is short, lasting only a few months, for the chum salmon, pink salmon and Chinook salmon as has been

mentioned before; in these species catadromous migration takes place in yearlings, whereas the parr stage is long, lasting 1 to 3 years, for sockeye salmon, coho salmon, steelhead, cherry trout, red-spot masu trout Oncorhynchus rhodurus and Atlantic salmon. Fishes belonging to the genera of the char and hutcho trout show a much weak catadromous trend, though vary to some extent depending on the latitude of their habitats and on the type of local populations. In nearly all salmonids catradromous migration takes place during a period of increasing daylight length, from spring toward early summer (excepting the red-spot masu trout which migrates from fall toward winter). Prior to catadromous migration salmonids must transform their bodies to adapt themselves to marine life. This transformation is called smoltification and is considered to be a kind of metamorphosis. Generally, parr develop into smolt, which are capable of adapting to seawater, in 3 to 5 months. In these smolt guanine and/or hypoxanthine is deposited onto the scales and subcutaneous tissues to turn their body surface to a lustrous and silver color as seen in such marine migratory fish as sardine and herring. Also blackening at the ends of the dorsal and tail fins, the phenomenon of which is called tsumakuro in Japanese, occurs[11]. Their vertebra elongates and the degree of fatness decreases, resulting in a smart body form as compared with parr. There is a strong possibility that such physiological and morphological changes are induced by growth hormone and thyroid hormone, i.e., it has been suggested in the cherry trout that secretion of these hormones is heightened by an increase in daylight length in the spring.

However, some individuals of cherry trout or red-spot masu trout, despite having

been born from the same parent, never undergo smoltification and spend their entire life in freshwaters (Fig. 5). When these individuals were p. 81 examined, nearly all of them were found to be prematurely developed males

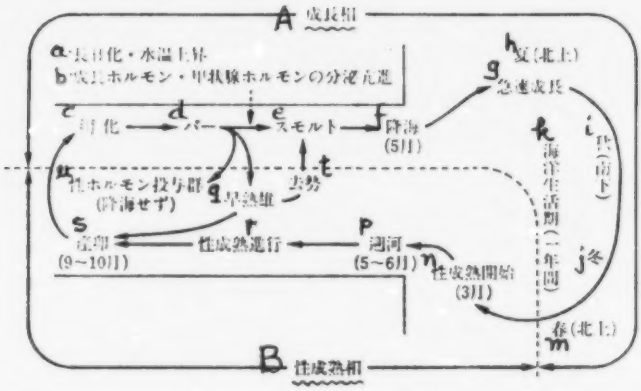

Fig. 5 Life cycle of cherry trout

A Growth phase
B Sexual maturation phase
- a daylight lengthening and rising water temperature
- b accelerated secretion of growth hormone and thyroid hormone
- c spawning
- d parr
- e smolt
- f catadromous migration (May)
- g rapid growth
- h summer (northward migration)
- i fall (southward migration)
- j winter
- k period of oceanic life (1 year)
- m spring (northward migration)
- n beginning of sexual maturation (March)
- p anadromous migration (May to June)
- q prematurely developed male
- r progression of sexual maturation
- s spawning (September to October)
- t castration
- u group of sex hormone administration (does not descend rivers to the sea)

with matured testis. In cherry trout generally smoltification occurs

from March to May and male fish that showed excellent growth during the summer of their yearling stage become sexually matured[12-14]; in August to September of the same year their testes are fully enlarged to fill the whole abdominal cavity and begin to release sperms. In such precocious males body color is darkened, the epidermis is thickened, and mucus cells are increased to form slimy skin; such males are called dark parr. In winter in these dark parr the testis shrinks and the ratio of gonads to body weight (GSI) declines and in the following spring GSI begins to increase again without undergoing smoltification.

From these observations it can be considered that those individuals which have sexually matured before undergoing smoltification cannot develop into smolt, i.e., cannot initiate catadromous migration. Hence in order to prove that sexual maturation inhibits smoltification, the present authors carried out castration operation on dark parr in August. Smoltification was observed in the following spring among those individuals from which the testes were completely removed. However, no smoltification was seen among any individuals for which castration operation was not successful and in which a piece or pieces of the testis even however small remained. When the blood level of male hormone was determined, about 1 ng/ml was detected in groups of parr that underwent no smoltification (a control group in which the abdominal cavity of fish was opened, but their testes were not removed and a group in which the removal of the testes was incomplete)[15]. It is suggested from the results of this experiment that sex hormones secreted from the gonad suppress smoltification. Accordingly, an experiment of administering several

hormones was conducted to ascertain which hormone would suppress smoltification (Fig. 6). To yearlings which are supposed to undergo

Fig. 6 Effect of hormone administration on smoltification in cherry trout

	(suppressive effect) smoltification suppression	increase in pituitary GTH content
methyl testosterone	+	+
testosterone	+	+
11-keto testosterone	+	+?
estradiol	+	+
dihydrotestosterone	-	-
	(stimulatory effect) seawater adaptability promotion	silvering of body surface
thyroxine	-?	+
growth hormone	+	±

smoltification given orally mixed with feed in spring were male sex hormones -- 17-alpha-methyl testosterone (MT), testosterone (T), 5-alpha-dihydrotestosterone (DHT) -- and a female sex hormone, estradiol-17-beta (E_2). Although smoltification appeared in a high percentage of fish in a control group, smoltification was completely suppressed as had been expected in those fish to which MT or T was administered similar to naturally occurring precocious males. In the MT- or T-administered fish, like dark parr, their body color darkened, their body height was high, the epidermis thickened, and mucus cells were seen to be increased. In these fish also elongation of the tail bone, a sudden increase in body length and a decline in fatness, all of which are associated with smoltification and were seen in the control group, were restrained. Development of

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seawater adaptability was also not seen in these hormone-administered fish. On the other hand, DHT, which has the strongest male hormone action in mammals, exhibited no suppressing effect on smoltification (Fig. 6). A point of further interest is that although under natural conditions nearly all female cherry trout undergo smoltification and only rare individuals remain in rivers, the female hormone of E_2, like MT or T, acted to suppress smoltification. In other words, the reason for the appearance of individuals that remain in rivers throughout their life in only male fish populations is likely to be that smoltification is not suppressed only by male sex hormones that are excreted from the testis, but that male fish tend to develop prematurely, whereas female fish take certain time to attain sexual maturation; E_2 concentration is unlikely to increase before the occurrence of smoltification. However, E_2 unlike MT or T lacked to effect thickening of the epidermis and increases in mucus cells; these two phenomena are considered to be specific to male hormones[16,17].

Next we examined whether smoltification that is caused by an artificial increase in day length would be suppressed by sex hormones as follows: yearling fish that do not undergo smoltification under natural conditions were reared under a short-day condition created with artificial illumination of 8L-16D (8-hour light followed by 16-hour darkness) beginning in June. This short-day condition was switched to a long-day condition of 16L-8D at the end of October and simultaneously oral administration of T or 11-keto testosterone (11-KT), which shows the strongest male sex hormonal effect in salmonids, was commenced. In December smolt that were equipped with complete seawater adaptability p.83

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appeared in the control group, but smoltification was again suppressed in the groups of T and 11-KT administration. This finding indicates that sex hormones suppress some part of a series of mechanisms responsible for the manifestation of smoltification[17].

Furthermore, we carried out an investigation to examine the season when sex hormones would be effective as follows: T was orally given to sexually immature yearling fish (excluding precocious males) from the end of September to the middle of December and the administered fish were observed for the occurrence of smoltification in the following spring. In the administered fish although their body color darkened, this hormone was found to have no influence on the appearance rate of smoltification in the following spring. This finding suggests that the action of T be transitory, i.e., when its concentration was elevated in the previous fall to even very high levels, but declines to low levels in the season of smoltification in spring, T has no suppressing effect. In naturally occurring precocious males their testes even shrunk in winter, yet are much larger than those of immature males and thus are considered to excrete an amount of male hormones that is sufficient to suppress smoltification (in the order of 1 ng/ml)[15].

Then by what mechanism(s) is smoltification suppressed by sex hormones ? The amount of gonadotrophic hormones (GTH) in the pituitary gland, when determined by radioimmunoassay, was found to be significantly higher in a group of fish in which smoltification was suppressed than in a control group or in a group of DHT administration. It has been reported in rainbow trout fry that E_2 and male sex hormones that are aromatizable

possess a positive feedback action of increasing the GTH content of the pituitary gland. In other words, there is a possibility that sex hormones induce a sexual maturation phase, resulting in the suppression of mechanism(s) responsible for the manifestation of smoltification. The fact that in nature sexual maturation never coincides with smoltification also appears to support this notion. It has also been reported in many salmonid fishes that the blood level of thyroid hormone (T_4) increases during smoltification[18-20]. The effect of T_4 on smoltification is said to be limited to promote the silvering of body color and to induce no development of seawater adaptability. The pattern of blood levels of T_4 in fish to which sex hormones were administered showed a definite decrease after MT, E_2 or 11-KT but no change after T. Since there is a report that T activates the secretion of T_4 from the thyroid gland, it is also highly probable that the thyroid effect varies with the type and dose of sex hormones administered. It has also been reported that elongation of the vertebra is remarkable in smolt[16] and that in some salmonid fishes the development of seawater adaptability is stimulated by growth hormone[20,21], indicating that the secretion of growth hormone is accelerated during smoltification. Hence one has to consider also the possibility of sex hormones acting on the hypothalamus-pituitary system to suppress the secretion of growth hormone or to modify the action of growth hormone peripherally. Furthermore, there are reports stating that sex hormones stimulate the secretion of prolactin from the pituitary gland[23,24]. Since prolactin controls freshwater adaptability and stimulates the secretion of mucus from the skin, the effects of these sex

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hormones are also likely to have been manifested through prolactin. Regardless of the possibilities mentioned above, many aspects of the mechanism by which sex hormones suppress smoltification remain to be answered.

In addition to cherry trout, precocious individuals appear also in the population of red-spot masu trout and Atlantic salmon. In the Atlantic salmon, however, it is known that some precocious fish undergo smoltification and descend to the sea in the following spring and that some parent fish that had ascended from the sea for spawning descend again to the sea and repeat anadromous migration in the following spawning season. These phenomena might be caused by the presence of a higher threshold value of sex hormones required for smoltification in Atlantic salmon than in cherry trout or by lower sensitivity to sex hormones in the former than in the latter, i.e., once the testis shrinks and the concentration of sex hormones declines, suppression of smoltification no longer works.

3.4.2 Spawning migration

There has so far been scarcely any report describing the type of signals by which salmonids initiate spawning migration. The fact that this migration takes place in such a wide sea area as the North Pacific Ocean may account for the difficulty with which such investigations can be accomplished. However, since spawning migration is a strongly directional movement to return for spawning to the rivers of birth, there will be no doubt that sexual maturation is the driving force of this migration. Chum salmon descend to the sea in about May and after spending about one month in bay areas travel northward then migrate for 2 to 4 years in the

northeastern Pacific Ocean including the Bay of Alaska. Adult fish travel northward to enter the Bering Sea in about June, appear in waters off the east coast of the Kamchatka Peninsula and come to Hokkaido in August to September (Fig. 7)[25,26]. In all of the returning fish, which vary in age, the gonads are in a matured state. On the other hand, cherry trout

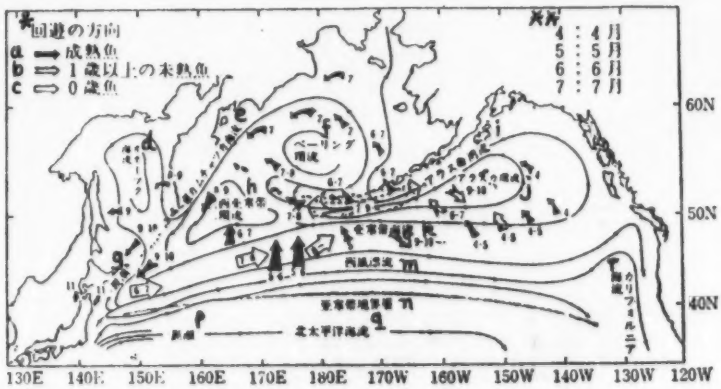

Fig. 7 Assumed routes of migration for chum salmon (Figure of Yonemori[25] was supplemented[26])

* Direction of migration
a mature fish
b immature fish, one-year old and older
c zero-year old fish

** 4: April
5: May
6: June
7: July

d Okhotsk current
e East Kamchatska current
f Bering return current
g Oyashio current
h West Subarctic zone return current
i Alaska Bay current
k Subarctic zone current
m West wind drift current
n Subarctic zone boundary layer
p Kuroshio current
q North Pacific current
r California current

smolt descend toward the sea in about May, travel northward to enter the Sea of Okhotsk, and in the fall begin southward migration to reach the southern part of the Sea of Japan in winter (Fig. 8)[27]. In the following spring they move northward and ascend from the sea in May to

July and spawn in the fall. At a glance there appears to be a difference

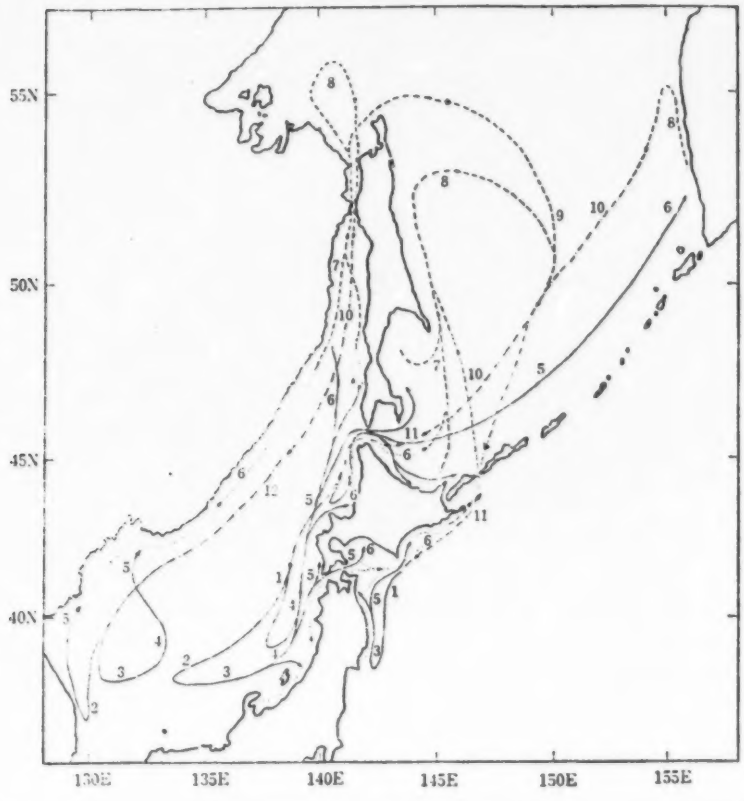

Fig. 8 Assumed routes of migration for cherry trout[27)]
Dotted lines: spring (April to June), broken lines: summer (July to September), chain lines: fall (October to December), solid lines: winter to spring (January to June)

in the season of anadromous migration between chum salmon and cherry p. 85
trout. However, this difference between the two species may be considered also to derive from different migratory distances: in contrast to chum salmon which are returning to rivers over a distance of several thousands of kilometers from the North Pacific, the anadromous migration of cherry trout takes place not far from the coasts of Japan. The time when sexual p. 86

maturation begins is March to April for cherry trout and chum salmon (Table 1)[28,29] and this appears to be true also for red-spot masu trout and pink

Table 1 Changes in the degree of sexual maturation in cherry trout that were caught from the Sea of Japan[28]

A 採捕月		B 性成熟度（生殖腺重量×100/体重）	
		C 雌	D 雄
a	3月 上旬	0.94	0.19
b	中旬	1.12	0.23
c	下旬	1.30	0.25
d	4月 上旬	1.64	0.26
e	中旬	2.02	0.36
f	下旬	2.25	0.44
g	5月 上旬	2.89	0.60
h	中旬	3.07	0.82
i	下旬	3.19	0.97
j	6月 上旬	3.05	1.11
k	中旬	6.35	1.38

A Month of catch
a March, early
b middle
c late
d April, early
e middle
f late
g May, early
h middle
i late
j June, early
k middle
B Degree of sexual maturation (gonad weight x 100/body weight)
C male D female

These values, when compared with those at the time of full sexual maturation, are considerably low, yet female values exceeded 1.0 from mid March onward, suggesting that yolk formation had begun.

salmon. This finding suggest strongly that in salmonids the gonads begin to develop in the spring of a year in which spawning migration is to take place and simultaneously spawning migration toward the rivers of birth starts. It would be most rational to consider these types of behavior as physiological phenomena that are effected through sex hormones. The relation between the manifestation of this migratory drive and sex hormones remains to be a topic of interest to be resolved.

Rousefell ranked various salmonid fishes according to their p. 87 catadromous migratory tendency and has found that the trend is stronger in more phylogenetically advanced fish species than in lesser ones[30]. It has been considered that during the ice age ancestors of salmonids had

gradually extended their living range from low-productive freshwaters to high-productive oceans. Nonetheless, today all of them, even those species with a strong catadromous migratory tendency, still spawn in freshwaters. In other words, it is likely that they will not (or are unable to) abandon freshwaters, especially rivers of their birth, as a spawning site. Accordingly, however wide they may extend their migratory range, they must return to the rivers of birth to be in time for spawning. Then would'nt it be reasonable to speculate that the start of sexual maturation triggers the spawning migration. Ability of individual fish that have matured in the sea to orient themselves to waters of the stream in which they were born and inability of those which have matured in rivers to descend to the sea are anyway essentially the same thing, which is likely to be controlled by sex hormones.

4. Conclusion

Heretofore spawning migration of several fish species has been outlined and most interesting in this description would be again a topic of what triggers spawning migration. It may safely be said that in the case of salmonids the start of sexual maturation acts as this trigger. Although sexual maturation of salmonids has been deemed to be induced by shortening daylight, indicating rapid accumulation of yolk, recent studies have revealed that early yolk accumulation has already begun in spring. That is to say, the blood level of sex hormones has begun to increase gradually from about this time and is likely to reach an amount that is sufficient to cause an urge to migrate for spawning. Those eels which are descending rivers to the sea are in an early stage of maturation and in

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this species the beginning of sexual maturation will be a prerequisite for spawning migration. In contrast to these species, for the aforementioned spawning migration behavior of Pacific mackerel, sardine, ribbon fish, croaker (Nibea argentata) and red sea bream Chrysophrys major almost nothing is known in this regard. These fish species are on a one-year cycle and migrate between north and south or between offshore wintering and coastal spawning sites, indicating a strong possibility in these species that fluctuation in water temperature rather than anything else acts as a trigger to initiate migration. Tuna of young ages migrate on a large scale across the oceans and when reaching a sexually matured age after a few years are assumed to take a return journey to rather limited spawning sites; this species is considered to be higher in ability to journey back than the aforementioned several species. Salmonids also migrate across the oceans and return to extremely limited spawning sites, i.e., rivers of their birth. The migratory behavior of these tuna and salmonid fishes, i.e., oceanodromous migration, the cycle of which is lifetime and which also includes small-scale migrations between north and south on a yearly cycle and spawning migrations only by those individuals that have begun sexual maturation, appears to support the presence of strong positive elements on the side of fish for the initiation of spawning migration (this can be called 'positive spawning migration'). As compared with this type of migration, the pattern of spawning migration for Pacific mackerel, sardine and ribbon fish may well be termed 'passive spawning migration', which is induced by fluctuation in water temperature. It would be desirable to elucidate further the physiological background of migration.

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7. Gonadotrophic hormone of migratory fishes (90)

By Susumu ISHII

1. Foreword

The relationship between reproduction and hormones is so deep that whenever hormones are mentioned, right away even experts, but non-experts in particular, conjure up the reproductive phenomenon. With vertebrates it is no exaggeration to say that all phenomena having to do with reproduction are invariably subject to regulation (endocrinal regulation) by hormones somewhere. Needless to say, endocrinological studies on the reproduction of fishes are also numerous, and cannot possibly be all introduced that simply. However, when it comes to studies dealing with reproduction and hormones involving the question of migration, the number drops abruptly.

Here, the study by the author and his co-researchers on the characteristics of the gonadotrophic hormone (GTH) of tuna well known as a migratory fish will be introduced in the main. Only, in discussing the GTH of tuna, the matter of the GTH of fishes in toto and even the GTH of vertebrates in toto cannot be ignored. Accordingly, in this chapter, we will first of all start with the GTH of mammals, then move on to the GTH in general in the lower vertebrates which include fish, and finally proceed to the GTH of yellowfin tuna now at issue.

We would feel rewarded if the readers might gain some knowledge of the characteristics of the GTH of tuna from this as well as learn how the researchers, faced with the huge and complex phenomenon called nature, were swung this way and that by their object of study and how they managed to somehow delve into just one tiny part of it and shed light on it.

2. Main research methods

2.1 Refinement and purification of hormone

The GTH of the various animals used here, except for the mammals, were mostly ones refined and purified by the authors et al. As methods, salting out by ammonium sulfate, ethanol precipitation, concanavalin A-cephalose adsorption (91)
chromatography, various ion exchanges and gel filtrations by normal column chromatography and high speed liquid chromatography, and reverse phase chromatography as well as isoelectric point electrophoresis and chromatofocusing and such have been used in various combinations. The fact that these combinations have been changing with the material used and the times is natural. The end products may all be said to be pure for all practical purposes.

The GTH of the mammals were provided from the National Institutes of Health (NIH) of the United States. Meanwhile, the amino acid composition of the GTH of yellowfin tuna was analyzed by co-researcher Dr. Hirokazu HAYASHI of the Endocrinology Research Institute of Gunma University.

2.2 Quantification of hormone

For the quantification of GTH, the radioreceptor assay (RRA) method utilizing the bonding reaction of GTH and its receptor has been used in the main. Also, while it is semi-quantitative, an in vitro method using the concentration rise of 3',5'-cyclic adenosinemonophosphate (cAMP) within the spermary as an indicator (cAMP method) was jointly used. Depending on the animal used as material, a method using the male hormone release from the spermary in vitro as an indicator (male hormone method) has also been used.

Let us elaborate on the RRA in a little more detail. The first step in which a hormone acts on a cell is where the hormone molecule bonds in a non-covalent bond to a certain protein molecule in the cell called a receptor. In the case of GTH, a receptor specific to it exists fronting on the outer surface of the protoplasmic membrane of the target cell. Unless it bonds with this receptor molecule, the hormone cannot manifest its function. Not only that, the various characteristics the hormone shows when acting on the cell are often none other than the characteristics of this receptor as well. In one instance of that there is what is called organ specificity of a hormone function. For example, the GTH is said to act only on certain specific cells in the spermary or ovary and never acts on other cells. This is because a specific GTH receptor exists only in those specific cells (GTH target cells) within the spermary or ovary. Also, there is a phenomenon called species specificity of a hormone function. That is, even if it is the same GTH, the GTH of another species of vertebrate ma not act on the gonad of a certain species of vertebrate or its effect may be weak. This is due to the fact that the characteristics of the GTH receptor of that animal species differ from the characteristics of the GTH receptor of the animal from which the GTH originated, and hence that GTH cannot bond or even if able to bond the affinity would be low. (92)

It was after a little while into the 1970s that the importance of this hormone receptor came to be recognized among researchers and studies of the receptor for GTH as well came to be conducted. Around that time, the author was confronted by one difficult problem. That was the development of an accurate as well as highly sensitive method of quantification of GTH. About this time, we were working on the purification of the GTH of chicken. But even though hormones thought to be pure were painstakingly produced from tons of chicken heads, when we sought to examine the purity from their biofunction we would end up using up virtually all the hard-won hormones. In particular, to specifically quantify

each of the two types of GTH in mammals, that is, the follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in those days a bioassay method of testing by injecting rats was regarded as the norm, so that an enormous amount of hormone used to be necessary. Of course, from around this time radioimmunoassay (RIA) had begun to be performed for the FSH and LH of rats. Regrettably, however, the antibody for the FSH and LH of rats does not indicate a cross-reaction with the FSH and LH of chicken, so this method could not be applied to chicken. Even to look at the reaction by injecting chicks required a considerable amount, so much so that research using lower amphibians and fishes much harder to obtain was thought to be close to impossible. Agonizing over this sort of problem one day, we suddenly hit upon the idea that a hormone receptor could perhaps be used in place of an antibody. The fact that RIA is possible is because there is a pure marked hormone and a specific antibody for that hormone. Birds react well to the FSH of mammals. Then naturally the FSH receptor of birds ought to bond well not only with the FSH of birds but with the FSH of mammals too. However, as long as it is a FSH receptor, it should not bond with LH or other substances containing hormones. Accordingly, we arrived at the belief that if we used marked FSH of rat and the receptor of quail or chicken, surely the FSH of chicken could be quantified in the same way as RIA. After much hardship, we succeeded in formulating a RRA capable of specifically quantifying the FSH and LH of chicken. After that, we formulated and used a variety of RRA using the coarse membrane fraction of the spermary of various vertebrates as the receptor and the GTH of a number of species of animals marked with ^{125}I as the marked hormone. While the sensitivity of RRA is lower than that of RIA, even so hormone quantities in units of ng can be detected. And because the species specificity is lower than with RIA, there is the convenience that hormones of other species of animals can also be quantified. (93)

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3. GTH of vertebrates and their receptors

What has been most thoroughly studied in terms of GTH and their receptors are naturally mammals. Of the GTH in mammals, FSH causes the development of eggs in the ovary, while in the spermary it stimulates the Sertoli cell and takes part in spermatogenesis. LH in the ovary causes matured eggs to be ovulated and luteinized, while in the spermary it causes the interstitial cell to fatten and the secretion of male hormones. It is also well known that in the ovary as well FSH and LH regulate the secretion of estrogens and corpora lutea. This FSH and LH are protein molecules that contain sugar in common with placental GTH or thyroid-stimulating hormone (TSH), with a molecular weight of around 30,000. Every hormone, no matter which, is a polymer formed by two different subunits called α and β being bonded together. It is also well known that while the α-subunit has a common chemical structure whatever the hormone, the β-subunit has a different chemical structure depending on the hormone. Consequently, it can be thought that the α-subunit contributes to the manifestation of the hormone function for cells, while the β-subunit contributes to hormone specificity (organ specificity if viewed in reverse). However, if dissociated into subunits, any hormone will stop manifesting its function. Furthermore, this α and β are coded by separate genes located in separate chromosomes, so it is thought they are each separately synthesized and later polymerize into a perfect hormone molecule. With respect to the FSH and LH of mammals, in a small number of species the amino acid configuration for the protein segment of each of their subunits has been made completely clear. For other vertebrates, however, there is nothing more than the amino acid configuration in some fishes (carp and chum salmon) having been reported.

The GTH of birds also consists of FSH and LH. Naturally, in birds and lower forms no placental GTH exists. Both hormones have a different amino acid composition than those of mammals, so that it is clear they have a different

chemical structure. The fact that they are glycoproteins and consist of two subunits, α and β, is the same as in mammals. In terms of their function relative to the spermary and ovary, for the spermary it has been well investigated and may be said to be virtually the same as in mammals. For the ovary there are few studies using the hormones of birds, but judging from the studies using the hormones of mammals, basically it is thought to be the same as in mammals. (94)

Now, studies on reptiles may be said to be virtually non-existent other than the study by a University of California (Berkeley) group, but it is known that FSH and LH likewise exist. In amphibians as well, FSH and LH have been refined and purified from bullfrogs by three research groups.[1-3] In the case of toads as well, FSH and LH were purified in the author's laboratory. Studies on the function of the FSH and LH of these poikilothermic vertebrates have primarily been done dealing with the spermary, and in every case the LH has a male hormone releasing function. Even if there were assumed to be this function in the FSH, it would be around 1/100 that of LH. While both hormones increase the cAMP in the spermary, LH is much more effective.

As above, to the extent that we have been able to purify and examine them thus far, it has become clear that two types of GTH molecules, FSH and LH, exist in all quadruped vertebrates. Now then, what about the receptors for these hormones? Two types consisting of a FSH receptor and LH receptor can be expected to exist, and in fact that is the case in mammals, each being known to bond specifically with FSH or LH only. Then, how do we go about examining this matter? For that, if it were the case of the FSH receptor of rat, marked rat FSH and a receptor fraction of the rat spermary (in reality the precipitation fraction at a centrifugation of around 10,000 x g of a homogenate of the spermary would be fine) would be reacted. Into that reaction solution would be added unmarked FSH or LH of various concentrations. After reacting until close to equilibrium, the radioactivity bonded to the receptor (in reality the centrifugated precipitate) is

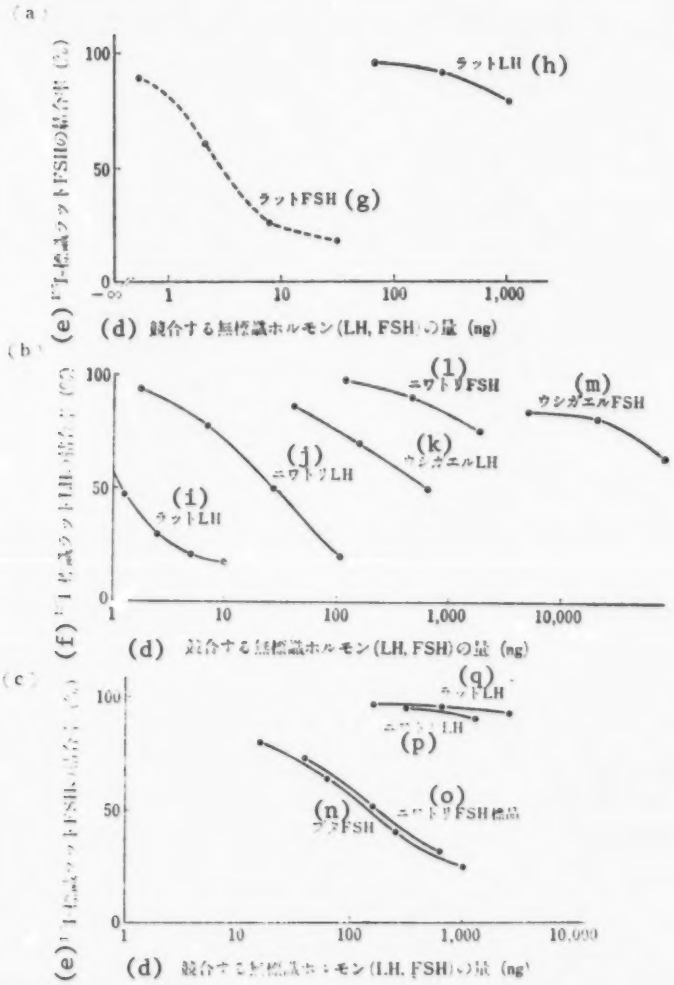

Fig. 1. Bond inhibition curves of FSH and LH to receptors showing hormone specificity of FSH receptors and LH receptors of mammals and birds. (a) Experiment using rat spermary receptor and marked rat FSH. (b) Experiment using rat spermary interstitial cell receptor and marked rat LH. (c) Experiment using quail spermary receptor and marked rat FSH.

(d) Quantity of competing unmarked hormones (LH, FSH) (ng)
(e) Bonding rate of ^{125}I-marked rat FSH (%)
(f) Bonding rate of ^{125}I-marked rat LH (%)
(g) Rat FSH
(h) Rat LH
(i) Rat LH
(j) Chicken LH
(k) Bullfrog LH
(l) Chicken FSH
(m) Bullfrog FSH
(n) Pig FSH
(o) Chicken FSH standard specimen
(p) Chicken LH
(q) Rat LH

measured. So doing, the radioactivity that has bonded should be lower depending on the concentration of FSH. That would be due to the fact that competition for a bonding site had occurred between the unmarked FSH and marked FSH. If this receptor bonds LH as well, then when LH is added as well the bonded radioactivity should diminish depending on its concentration. What we actually undertook is presented in Fig. 1a. The LH even at fairly high concentration virtually does not suppress the bonding of marked FSH. Contrary to that, the FSH suppresses it well. The same thing undertaken using rat LH as the marked hormone is presented in Fig. 1b. Rat LH has specifically suppressed the marked LH of rat. From these bonds, it can be seen that in rat the FSH receptor bonds FSH only while the LH receptor bonds LH only. When the same thing was undertaken using the FSH and LH (96)
of chicken and the receptor fraction of the spermary of quail, the same results were obtained (Fig. 1c), so it can be seen that in birds as well independent receptors of FSH and LH each exist separately.

Next, the same experiment was undertaken using the FSH and LH of bullfrog and the coarse membrane fraction of its spermary.[4] Upon using bullfrog FSH as the marked hormone and both the FSH and LH of bullfrog as unmarked hormones, unlike the mammals and birds, not only the FSH but even the LH suppressed the bonding of marked FSH exactly the same way (Fig. 2a). This indicates that the LH of bullfrog can bond in exactly the same place seemingly in exactly the same way as FSH. That is, the FSH receptor of bullfrog cannot distinguish between (97)
FSH and LH. Thereupon, this time we changed things around and examined the LH receptor using bullfrog LH as the marked hormone. In this case the situation changed slightly. Although the LH suppressed the bonding of marked LH at a fairly low concentration the same as in the previous experiment, the FSH required a higher concentration, and the curves indicated by the two were no longer parallel. Even at that, however, the fact was elicited that FSH can still bond to a receptor of LH as well. In other words, it means there is only one type of

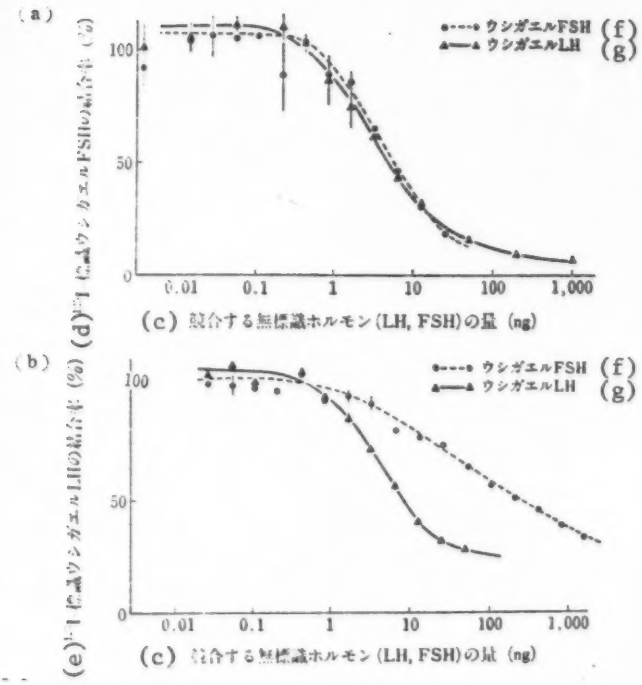

Fig. 2. Experiment showing that GTH receptor of bullfrog will bond both FSH and LH. (a) Bullfrog spermary receptor and marked bullfrog FSH were used. (b) Bullfrog spermary receptor and marked bullfrog LH were used.

(c) Quantity of competing unmarked hormones (LH, FSH) (ng)
(d) Bonding rate of ^{125}I-marked bullfrog FSH (%)
(e) Bonding rate of ^{125}I-marked bullfrog LH (%)
(f) Bullfrog FSH
(g) Bullfrog LH

GTH receptor in bullfrog, and both FSH and LH will bond there. This is thought to sort of contradict the fact that FSH and LH have different functions relative to the spermary. However, results indicating that we cannot necessarily say so are known with mammals. LH whose sugar has been artificially removed will bond to a LH receptor with the same or a higher affinity than before the removal, but such biofunctions as raising the cAMP concentration or causing the release of sex steroid hormones are absolutely not manifested. That is, the bonding site vis-a-vis the receptor and the site of adenylcyclase activation are different, and it is thought that by sugar removal the former is not affected but the latter had been ruined. Consequently, even if the FSH of bullfrog bonds to the

interstitial cell of the bullfrog, the FSH of the bullfrog probably cannot induce the activation of adenylcyclase. In fact, the cAMP accumulation function is considerably weaker with FSH. However, a problem still remains. FSH and LH are often both secreted at the same time. Then, it is highly possible that FSH gets in the way of the bonding of LH to the GTH receptor. However, as in Fig. 2b, to suppress the bonding of LH there has to be a fairly high concentration of FSH, so that this phenomenon can be understood as being due to a mechanism whereby, if FSH exists, the affinity of the receptor to LH will increase depending on the concentration of FSH. In this way, the bonding of LH gets hard to be inhibited by FSH, so that even if both coexist the function of LH would get amply manifested.

In this way, it became clear that in amphibians even if there are two types of hormones there is only one type of receptor and this one receptor itself has the property of distinguishing and reacting with both hormones. Now then, what about this question of two types of GTH in fishes?

4. GTH of fishes (98)

Studies undertaking the refinement of GTH in fishes are most numerous dealing with the salmon family, and at the very least there are nine on the genus Oncorhynchus,[5-13] one dealing with rainbow trout[14] and one dealing with Coregonus.[15] Others are on carp,[16] silver bighead (Leuciscus molitrix (Valenciennes)),[17] Tilapia,[18] pike eel,[19] catfish,[20] sturgeon,[21] Amia,[22] and northern dogfish (Squalus acanthias (Linné)),[23] plus there is the study on yellowfin tuna[24] taken up here.

If we summarize the studies up to now, the GTH of fishes, like other vertebrates beginning with mammals, is likewise a glycoprotein consisting of two subunits, with an amino acid composition resembling others to a certain extent but clearly different as well. According to a study on the GTH of carp which clarified the amino acid configuration in parts and compared it to that of mammals,[25] with respect to the α-subunit, of the 24 residues between the

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10th - 33rd ones from the N-end, the ones in common with those of cow and sheep come to 18 residues (75%). Also, the Tyr-Tyr-His-Ser-OH configuration on the C-end is totally in common with mammals as well. As for the β-subunit, on the other hand, if we compare the amino acid residues in position 11 - 25 from the N-end with human LH-β, 11 of the 16 residues are the same, whereas with human FSH-β no more than seven of the 16 residues are the same. Also, while LH-β of mammals is held to be characterized by a high proline content (11 - 17%), GTH-β of carp is likewise high in this proline content (11%), and from these points the GTH of carp may be said to resemble the LH more than the FSH of mammals. However, whether this matter of proline can be said to apply in general to fishes in toto is still questionable as discussed later.

When all is said and done, however, the most important question at present with regard to the GTH of fishes would probably be the question of whether two types of hormones corresponding to FSH and LH exist or not. On this point, even in various studies on fishes by the majority of researchers, up to now basically only one type of GTH had been isolated. This was so despite the variety of methods used in assaying its bioactivity, such as the cAMP method, intake of ^{32}P into the gonad, sex steroid releasability, gonadal weight increase and RRA. Also, a group in France which used the GTH of carp has stated that with this one type of GTH alone the function of the ovary can be completely fulfilled. On the other hand, Idler's group[26] in Canada has reported that in the GTH of salmon are GTH-I which contains little or no sugar and GTH-II which contains sugar, both of which not only differ in amino acid composition but differ greatly in biological function as well. Specifically, GTH-I is said to primarily have the (99) function of acting on immature eggs and stimulating the intake of yolk, while GTH-II has the function of acting on mature eggs and causing them to ripen and be ovulated. From this fact, the former is called vitellogenic gonadotropin and the latter maturational gonadotropin. However, there does not yet appear to be

anyone who has followed up this study. Only, according to a recent study by people in France,[27] when GTH containing sugar of carp is isolated by the same method as Idler et al., a fraction corresponding to Idler's GTH-I containing no (or little) sugar of about 5% of the total is produced. Also, they have stated that there is ample yolk accumulability in the GTH with much sugar, so that this study, while negative, is thought to seem to be refuting the thinking of Idler et al.

5. Research focusing on GTH of yellowfin tuna

At the beginning of research on the GTH receptor of lower vertebrates, the author et al. discovered the fact that, while the FSH of rat virtually does not bond to the gonad of bullfrog or toad, it bonds extremely well to the spermary of Xenopus. A further interesting fact is that the LH rather than FSH of chicken, bullfrog and even toad bonds better to the rat FSH receptor of this Xenopus (Fig. 3).

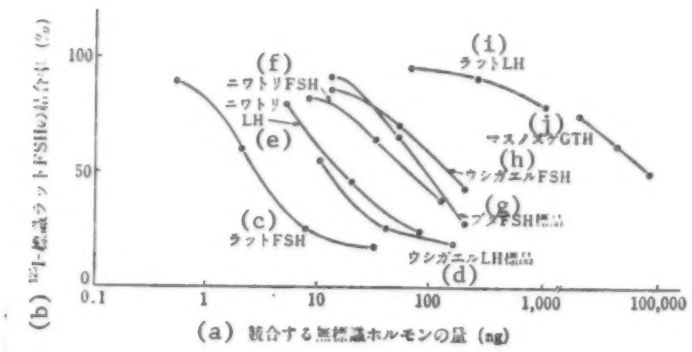

Fig. 3. Experiment using the receptor of Xenopus spermary and marked rat FSH to see what sort of hormones of what kind of animals can bond to this receptor.

(a) Quantity of competing unmarked hormones (ng)
(b) Bonding rate of ^{125}I-marked rat FSH (%)
(c) Rat FSH
(d) Bullfrog LH standard specimen
(e) Chicken LH
(f) Chicken FSH
(g) Pig FSH standard specimen
(h) Bullfrog FSH
(i) Rat LH
(j) Chinook salmon GTH

(100)

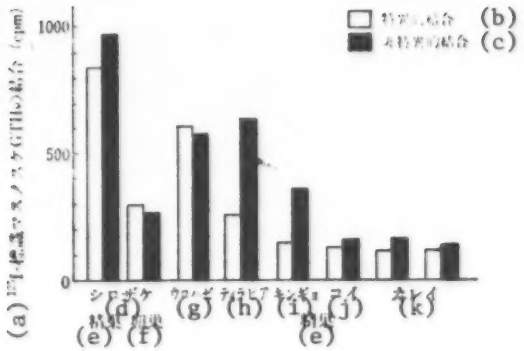

Fig. 4. Experiment marking chinook salmon GTH to see the spermary of which kind of fish it bonds well to.

(a) Bonding of ^{125}I-marked chinook salmon GTH (cpm)
(b) Specific bond
(c) Non-specific bond
(d) Chum salmon
(e) Spermary
(f) Ovary
(g) Glossogobius olivaceus (Temminck & Schlegel)
(h) Tilapia
(i) Goldfish
(j) Carp
(k) Plaice

Later on, it was also noticed that the pituitary extracts of chinook salmon, particularly their GTH fraction, also bond to this receptor. Thereupon, using RRA based on receptors, we succeeded in isolating nearly pure GTH from the (101) pituitary gland of chinook salmon.[11] This end product has a strong elongating function on the ovipositor of Acheilognathus moriokae and the function of causing the release of estrogen from the ovary of the salmon family, and was clearly identified as being GTH. Next, upon marking this GTH of chinook salmon and examining its specific bond to the spermary of various species of fish, it became clear that, outside of the salmon family, it bonds well to the spermary of Glossogobius olivaceus (Temminck & Schlegel), and that, to this chinook salmon GTH receptor in the spermary of Glossogobius olivaceus (Temminck & Schlegel), the GTH fraction of various fishes, such as carp and tuna for example, indicates bondability as well (Fig. 5a). Thereupon, using this kind of RRA, the refinement,

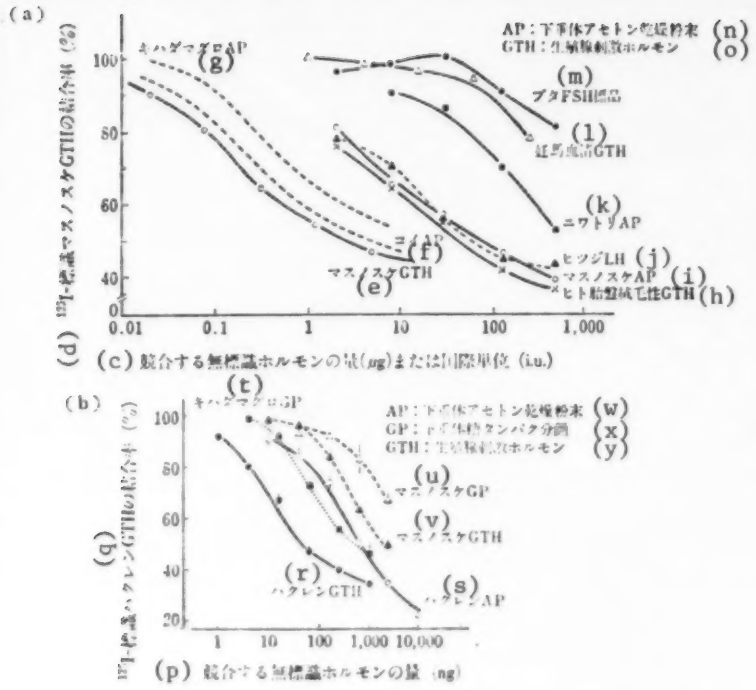

Fig. 5. (a) Experiment on the bonding of marked chinook salmon GTH to the spermary of Glossogobius olivaceus (Temminck & Schlegel) to see the GTH of which kind of animals compete.
(b) Experiment on the bonding of marked silver bighead GTH to the spermary of Glossogobius olivaceus (Temminck & Schlegel) to see the GTH of which kind of fish compete.

(c) Quantity of competing unmarked hormones (μg) or international units (i.u.)
(d) Bonding rate of ^{125}I-marked chinook salmon GTH (%)
(e) Chinook salmon GTH
(f) Carp AP
(g) Yellowfin tuna AP
(h) Human placental ciliary GTH
(i) Chinook salmon AP
(j) Sheep LH
(k) Chicken AP
(l) Pregnant mare serum GTH
(m) Pig FSH standard specimen
(n) AP: Pituitary gland acetone dried powder
(o) GTH: Gonadotrophic hormone
(p) Quantity of competing unmarked hormones (ng)
(q) Bonding rate of ^{125}I-marked silver bighead GTH (%)
(r) Silver bighead GTH
(s) Silver bighead AP
(t) Yellowfin tuna GP
(u) Chinook salmon GP
(v) Chinook salmon GTH
(w) AP: Pituitary gland acetone dried powder
(x) GP: Pituitary glycoprotein fraction
(y) GTH: Gonadotrophic hormone

purification and formulation of RIA of the GTH of silver bighead were carried out at the Hanyu Laboratory of Tokyo University.[17] Upon marking this GTH of silver bighead and letting it bond to the spermary of Glossogobius olivaceus (Temminck & Schlegel), it indicated an extremely high bondability (Fig. 5b), revealing that even with this system of RRA the GTH of yellowfin tuna and chinook salmon or the fractions containing them can be quantified.

Now, the refinement of the GTH of yellowfin tuna was first of all undertaken using this RRA. The material was collected from adult yellowfin tuna that had been frozen. The pituitary gland, after being preserved in cold acetone intact, was turned into dried powder, the purification of which was done by the same method as that described earlier in the section on hormone refinement and purification. Along the way, to see in which fraction there was GTH activity was naturally examined with a system of RRA of silver bighead GTH and Glossogobius olivaceus (Temminck & Schlegel) spermary, while terminally this was fractionated using an anion exchange column called Mono Q. The effluence pattern of protein when a high speed liquid chromatography apparatus was used is as shown at the top of Fig. 6. In all, peaks large and small numbering as many as 20 can be seen. The GTH activity examined by RRA shows up in the form of four peaks, 2, 3, 4, 5, with shoulders on the right side roughly speaking. As can be seen by looking at Fig. 6a and b in contrast, the GTH activity (specific activity) per protein weight examined by this RRA is highest in fraction 5 and lowest in fraction 2. In fraction 1 there was not activity enough to be detectable. Accordingly, for fractions 2 - 5 the biological activity by the cAMP method relative to the spermary of Glossogobius olivaceus (Temminck & Schlegel) was examined. Consistent with the RRA activity, fraction 5 was the highest in specific activity and fraction 2 the lowest. In other words, the RRA activity and the biological activity were parallel, so these fractions were judged to be GTH.

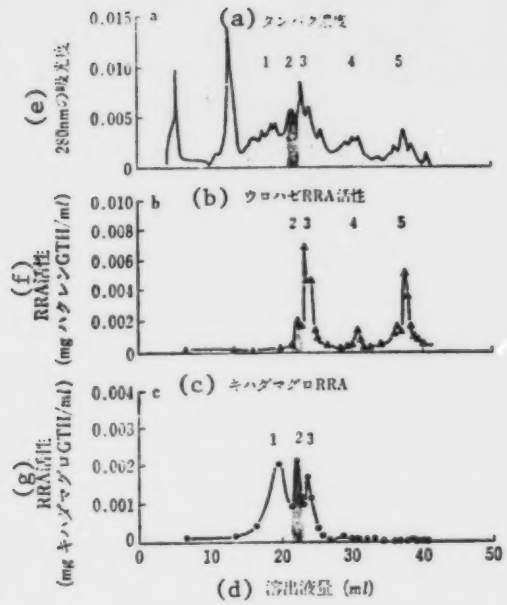

Fig. 6. Effluent pattern of yellowfin tuna pituitary extracts in anion exchange of high speed liquid chromatography. Top shows protein quantity at light absorption of 280 nm. Centre is the pattern of Glossogobius olivaceus (Temminck & Schlegel) spermary RRA activity. Bottom is the pattern of yellowfin tuna spermary RRA activity.

(a) Protein concentration
(b) Glossogobius olivaceus (Temminck & Schlegel) RRA activity
(c) Yellowfin tuna RRA
(d) Effluent quantity (mL)
(e) Absorbance at 280 nm
(f) RRA activity
(mg silver bighead GTH/mL)
(g) RRA activity
(mg yellowfin tuna GTH/mL)

Furthermore, marking this fraction 2 and 5 with ^{125}I, we examined whether or not they would bond to the coarse membrane fraction of the spermary of yellowfin tuna and Glossogobius olivaceus (Temminck & Schlegel) (Fig. 6c). However, fraction 5 which had the highest activity in the spermary of Glossogobius (102)
olivaceus (Temminck & Schlegel) whether by RRA or by biological assay, indicated absolutely no bond to the spermary of yellowfin tuna. Conversely, fraction 2 bonded far better to the spermary of yellowfin tuna than the spermary of Glossogobius olivaceus (Temminck & Schlegel). In that the procurement of live

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yellowfin tuna was difficult, the spermary of mackerel in the same family was used and the bioactivity of fractions 2 - 5 by the cAMP method was examined. Thereupon, it was found that fractions 2 and 3 which were rather ineffective on Glossogobius olivaceus (Temminck & Schlegel) had a high biological activity relative to mackerel, but fractions 4 and 5 which were highly effective on Glossogobius olivaceus (Temminck & Schlegel) were absolutely ineffective on mackerel spermary. In short, in yellowfin tuna exist 1) GTH which is effective on Glossogobius olivaceus (Temminck & Schlegel) but absolutely ineffective on the tuna family (fractions 4 and 5), and 2) GTH which is effective on both the tuna family and Glossogobius olivaceus (Temminck & Schlegel) (fractions 2 and 3). Thereupon, since there was the possibility that a third GTH exists, that is, a GTH which is effective on the tuna family but absolutely ineffective on Glossogobius olivaceus (Temminck & Schlegel), the entire lot including fraction 1 which had hitherto been ignored was re-examined by RRA using a marked fraction 2 and yellowfin tuna spermary. As a result of that, high RRA activity relative to yellowfin tuna was detected in fraction 1 which had hitherto been absolutely ineffective on (103) Glossogobius olivaceus (Temminck & Schlegel). The specific activity of this fraction by yellowfin tuna RRA is clearly higher than fraction 2 or 3.

Let us consolidate the foregoing results.

1) In the pituitary gland of yellowfin tuna exist glycoproteins thought to be five types of GTH having differing mobility in anion exchange chromatography.

2) Fraction 1 which is thought to have the lowest activity among them indicates the highest bondability to the GTH receptor in the spermary of yellowfin tuna. But it cannot bond at all to the GTH receptor in the spermary of Glossogobius olivaceus (Temminck & Schlegel).

3) Fractions 5 and 4 thought to have the highest acidity have, contrary to fraction 1, high bondability to the GTH receptor in Glossogobius olivaceus (Temminck & Schlegel) spermary, but cannot bond to the GTH receptor in yellowfin

tuna spermary. It is also the same with the bioactivity by the cAMP method.

4) The two intermediate fractions (2 and 3) can bond to the receptors of both fish, and in terms of bioactivity they raise the cAMP concentration in the spermary of both fish. However, fraction 2 is more effective on tuna, while fraction 3 is more effective on Glossogobius olivaceus (Temminck & Schlegel).

Fraction 1 and fractions 4 and 5 are clearly different GTH. The former may be called tuna type GTH and the latter two Glossogobius olivaceus (Temminck & Schlegel) type GTH. The possibility of fractions 2 and 3 being mixtures of fractions 1, 4 and 5 cannot be totally denied. Now, the amino acid composition of this fraction 1 and 5 and the amino acid composition of the GTH of various other fishes have been shown in Table 1. Also, while in just a preliminary way, fraction 1 was divided into subunits and their amino acid composition was examined as well, but unlike the case of carp, the proline content of the β-subunit was approximately 6% and not that high. From this point anyway, this β cannot be said to resemble LH-β of mammals.

With a system of RRA using yellowfin tuna spermary and fraction 2 that was marked, the bondability of the GTH and pituitary glycoprotein fraction of various fish species to the GTH receptor of yellowfin tuna was examined as well (Fig. 7). The interesting fact was that this yellowfin tuna GTH receptor was extremely high in species specificity and did not indicate a bond with the GTH of chinook salmon or silver bighead or bullfrog. Neither, of course, will it bond with the GTH of mammals or birds. It did, however, indicate high bondability to the pituitary extracts of several species of tuna other than yellowfin tuna in the mackerel family.

Table 1. Amino acid composition of GTH of fishes (104)

(a) サケ科	Breton *et al.* (1976) (d) ニジマス	Pierce *et al.* (1976) (e) マスノスケ	Yoneda *et al.* (1977) (e) マスノスケ	Idler & Ng (1979) (e) マスノスケ	
				Con-A-I	Con-A-I
Lys	5.7	5.7	4.4	8.7	6.5
His	3.7	1.6	2.2	2.2	2.9
Arg	4.0	2.7	3.1	3.9	3.2
Asp	10.3	11.3	10.0	10.2	8.9
Thr	8.8	11.3	8.6	6.0	14.7
Ser	5.1	6.5	7.3	6.5	9.7
Glu	9.1	9.7	13.3	8.6	5.3
Pro	6.7	7.0	8.7	4.8	8.9
Gly	4.3	5.7	5.8	6.8	3.8
Ala	3.4	3.2	4.2	9.1	2.1
1/2-Cys	7.0	11.9	8.0	4.0	6.1
Val	7.1	5.9	7.0	8.0	8.1
Met	2.2	2.4	2.6	2.1	1.5
Ile	4.1	4.0	4.4	4.7	4.8
Leu	5.8	4.6	6.7	8.4	6.5
Tyr	2.9	3.8	1.1	1.7	4.0
Phe	3.1	2.7	2.6	8.0	3.1

(b) コイ科	Burzawa-Gerard & Fontaine (1972)	Kobayashi *et al.* (1985)	Idler & Ng (1979)	
	Cyprinus carpio	*Hypophthalmichthys molitrix*	コイ (f)	
			Con-A-I	Con-A-II
Lys	6.7	5.7	6.0	4.3
His	3.2	3.7	4.8	2.8
Arg	4.1	4.8	5.5	3.8
Asp	8.4	8.2	12.6	9.2
Thr	7.7	8.4	5.6	6.2
Ser	6.8	11.7	7.1	8.5
Glu	9.2	8.2	11.3	9.6
Pro	8.3	7.5	6.2	6.5
Gly	3.9	7.7	6.4	7.0
Ala	3.8	4.9	4.9	3.8
1/2-Cys	6.9	4.2	4.3	6.0
Val	11.9	8.1	5.6	4.2
Met	1.5	0.9	2.1	2.8
Ile	2.8	2.9	3.5	4.3
Leu	7.4	5.6	10.2	8.8
Tyr	4.8	4.4	2.9	6.0
Phe	2.8	3.1	3.3	6.3

(c) その他	Farmer & Papkoff (1977) (g) ティラピア	Hung *et al.* (1985) Pike eel	Ng & Idler (1979) Plaice		著者ら (h) (i) キハダマグロ		
			Con-A-I	Con-A-II	Fr. 1	Fr. 2	Fr. 5
Lys	4.4	5.2	7.3	5.9	5.3	5.4	5.7
His	2.8	2.6	3.5	2.8	4.6	5.1	4.7
Arg	3.8	5.8	4.7	3.1	4.0	3.6	3.5
Asp	10.0	9.7	11.7	11.3	9.3	9.5	9.5
Thr	6.7	7.4	6.3	6.9	8.4	8.7	9.1
Ser	7.7	8.6	6.9	7.7	7.3	6.8	6.2
Glu	10.7	8.5	10.4	8.9	9.5	9.5	8.8
Pro	8.0	8.4	4.7	4.4	6.2	7.6	8.3
Gly	7.1	3.8	8.1	9.9	7.4	5.5	4.2
Ala	7.3	3.4	7.0	6.8	4.2	4.4	4.3
1/2-Cys	3.5	8.9	1.0	4.2	(3.8)	(3.6)	(3.8)
Val	6.8	7.2	6.0	6.4	6.6	6.9	7.1
Met	2.9	1.5	2.0	7.4	2.1	2.4	2.8
Ile	5.3	3.9	4.2	3.7	6.6	5.5	5.1
Leu	6.7	6.1	8.8	7.1	6.0	5.9	5.6
Tyr	3.2	5.1	2.7	2.4	5.9	5.2	5.8
Phe	3.3	3.6	4.6	4.1	3.2	4.5	5.4

(a) Salmon family
(b) Carp family
(c) Others
(d) Rainbow trout
(e) Chinook salmon
(f) Carp
(g) Tilapia
(h) Study by author et al.
(i) Yellowfin tuna

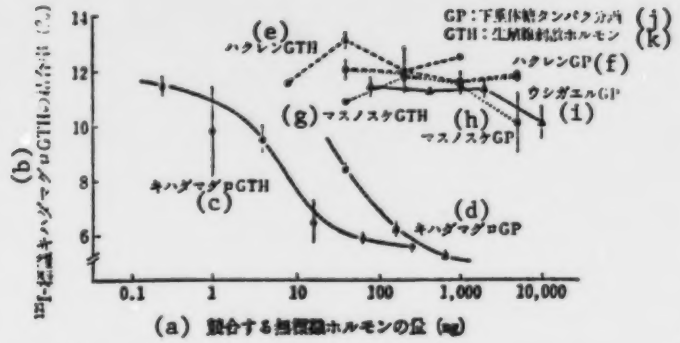

Fig. 7. Competitive inhibition experiment to examine the bonding of GTH of other fishes to the GTH receptor in yellowfin tuna spermary.

(a) Quantity of competing unmarked hormones (ng)
(b) Bonding rate of ^{125}I-marked yellowfin tuna GTH (%)
(c) Yellowfin tuna GTH
(d) Yellowfin tuna GP
(e) Silver bighead GTH
(f) Silver bighead GP
(g) Chinook salmon GTH
(h) Chinook salmon GP
(i) Bullfrog GP
(j) GP: Pituitary glycoprotein fraction
(k) GTH: Gonadotrophic hormone

6. Molecular multiformity of fish GTH

The GTH originating in the pituitary gland or placenta of vertebrates consists of numerous (at least three) molecular types which, although the same biologically or immunologically, differ slightly in isoelectric point with each other. In other words, it is well known that there is a molecular multiformity called microheterogeneity. In recent studies this is sometimes said to be due to differences in the content level of one part of sugar (sialic acid), but the notion has emerged that it is due to the fact that slight differences occur in the protein segment of the β-subunit after hormone molecule synthesis. Even in the GTH of fishes this kind of molecular multiformity has been reported in silver bighead, salmon, sturgeon and pike eel. Consequently, certain of the GTH of yellowfin tuna may be attributable to this type of molecular multiformity. However, one would be hard pressed to say that the difference between fraction 1

and 5 is of this type if one examines the amino acid composition and studies the differences.

The GTH of chum salmon has also been reported to be separable into two types by electrophoresis,[13] but in that both of them have the activity to cause gonadal weight to increase or cause estrogen to be secreted from the ovary, they may instead correspond to the difference between fractions 1 and 2 or 3 of yellowfin tuna.

7. Reason why GTH ineffective on tuna exists in tuna

Why does a substance which seems to be effective as GTH on Glossogobius olivaceus (Temminck & Schlegel) but is absolutely ineffective on tuna exist in the pituitary gland of tuna? Judging from the results up to now, there is a (106)
possibility that this kind of substance also exists in silver bighead and chinook salmon as well. Either way, in the case where multiple GTH molecules exist, two possibilities can be considered. One is the possibility that they are, similar to the relationship between FHS and LH, coded with respectively different structural genes or at different gene segments. The other is that what had been coded with identical genes had, after synthesis, split into a multiple of types due to segmental severance or differences in the way sugar attached and such. If the former possibility were assumed to be correct, this existence of multiple GTH molecules may be indicating an evolution of the GTH molecules of tuna. In mammals, the fact that a neuroderm hormone of lower vertebrates called arginine vasotocin shows up at least in the embryo stage is likewise probably indicating molecular evolution. Again, the question would arise as to why an absolutely useless molecule would develop and exist in tuna. However, it is known that in the neuroderm hormone as well, substances whose role is biologically unclear like mesotocin or isotocin do exist in lower vertebrates. Consequently, it is not that odd for Glossogobius olivaceus (Temminck & Schlegel) type GTH to be present in tuna. It could be that this had been a hormone molecule of tuna in

the past, or conversely it could also be considered that Glossogobius olivaceus (Temminck & Schlegel) came to utilize this meaningless substance as a new hormone. However, rather than indulging in such fancy, it goes without saying that research by setting forth good hypotheses and steadily corroborating them is necessary. And from such research, there could some day emerge a tie-in between this study and the phenomenon called migration.

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CHAPTER 8: FINAL MATURATION OF EGGS AND SPERMATOZOA

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by Yoshitaka NAGAHAMA

1. FORWARD

The parent salmon who has arrived at the spawning ground after overcoming various kinds of obstacles remains at that site for awhile waiting for a chance to reproduce. This is the preparatory period so that the fertilization as a result of the union of the female and male gametes can take place in a normal manner. From the perspective of the female, it is the time when the egg cells in the ovary are discharged out of the follicle (ovulation) after it has attained fertility (egg maturation). Normally, the division of the egg cells in the ovary is suspended immediately after the commencement of the first maturation division (meiosis), and this state is maintained for a long period of time from the egg yolk formative period to immediately before ovulation. The eggs in such a state are not fertile. The egg cells having attained

large nuclei (germinal vesicles) and accumulated sufficient yolks eventually recommence the maturation division triggered by the stimulation of appropriate hormones, and, after going through the decaying of the germinal vesicle and the release of the first polar body, they again stop dividing at the point when the middle stage of the second maturation division has been reached. At this point, the eggs are still in their follicle but when the insemination takes place they can be fertilized in a normal manner and embryogenesis can commence. Consequently, these eggs are mature unfertilized eggs. Strictly speaking, the egg maturation in the vertebrates means that egg cells complete the maturation division twice and with their nuclei becoming the female pronuclei they enter the state in which they can be fused with the male pronuclei formed by the transformation of the spermatozoa which have penetrated to the interior of the eggs. However, in general, the process from the recommencement of the first maturation division triggered by the hormonal stimulation of the immature eggs to the stage where the middle period of the second maturation division is arrived at thus making them fertile in their follicle is often called egg maturation.

On the other hand, pronounced physiological changes in the testes take place in males during the spawning period. For example, in spite of the fact the testes of dog salmon caught at the mouth of the Ishikari River are full of spermatozoa that are fully formed morphologically, these

spermatozoa have no motility physiologically and even when their abdominal part is pressed the spermatozoa cannot be squeezed out of the body. In this manner, the spermatozoa with motility are only obtained when the salmon reaches the spawning ground after climbing the river.

As described here, even after salmon have reached the 109
spawning ground, an important process of the final maturation of female and male gametes still remains to be completed before a normal fertilization can be realized. When the river water is contaminated or when humans attempt to control the reproduction of fish, unexpected damage sometimes occurs somewhere within this process. Needless to say, in this instance no fertilization takes place, therefore, no birth of new life. Consequently, research to clarify the mechanisms of the final maturation of the male and female gametes aids greatly in the establishment of the method of control for the maintenance of the variety of useful fish resources and their reproduction by artificial means. Furthermore, such research using salmonids as the subject have come into attention in recent years in the field of basic reproductive biology. The reason for this lies in that salmonids are a uniquely valuable subject for this kind of research in addition to the fact that the chemical properties and the action mechanisms of the effective hormones have much in common with those found in higher animals including the mammals. Of the migratory fishes, research is especially advanced in the case of salmonids. For

this reason, this article focuses on this family of fishes and the control mechanisms of the internal secretion during the final maturation of eggs and spermatozoa will be outlined.

2. EGG MATURATION

When the field of study concerning egg maturation and ovulation in fishes was still in the early stage of development, the methods used in research consisted entirely of those in which various gonadotropic hormones (GTH) and steroid hormones were injected into the abdominal cavity, however, soon with the development of the *in vitro* cultivation methods using the testis and ovarian follicle these tissues were being used as the specimen for bioassay and since that time the research on the subject of egg maturation has made a rapid progress. The use of these methods enabled one to examine the effect of various hormones in inducing egg maturation in several species of teleost including salmonids. However, regardless of whether the study is conducted *in vivo* or *in vitro* situations, it is not possible, of course, to conclude that the hormone being administered is the one inducing egg maturation in that fish on the sole basis that this externally added hormone had apparently caused the effect of inducing the egg maturation. Consequently, there is a need for the uncovering of true intrinsic factors affecting the induction of egg maturation but this is not very easy to do. In order to isolate a physiologically active substance and to determine its chemical structure, a sufficient quantity of starting material and

simple but highly sensitive bioassay are required. On this point, the salmonoid ovarian follicle was the optimum specimen for the isolation and identification of egg maturation inducing hormones. Some of the reasons for this include the fact that, as explained earlier, a large number of ovarian follicles which are just prior to maturation can be obtained since the development of ovarian follicles is synchronous as well as to the fact that because each individual ovarian follicle is large, a large amount of egg maturation inducing hormones are excreted by the reaction with GTH in in vitro conditions. Futhermore, in this article, the term "ovarian follicle" is used to denote a structural unit consisting of one egg cell and the somatic cell follicular tissue surrounding it. 110

2.1 The Trigger of Egg Maturation

When a salmonoid ovarian follicle whose yolk has been completely formed and has sufficiently grown is put in a dish and cultured with GTH refined from dog salmon, the egg cell matures. Consequently, as in other vertebrates, the hormone that plays the role of the trigger in causing the egg maturation in salmonids is GTH secreted by the pituitary gland[1)-3)]. On the basis of the findings of past research concerning GTH of the teleost, the glycoprotein GTH (maturational GTH) of a molecular weight of 25,000 to 40,000 is considered to be involved with the induction of egg maturation[4)]. This egg maturation inducing effect of GTH is not observed with the egg cell in an exposed state as a result

of the removal of follicular tissue. However, even such an exposed egg matures normally if it is cultured in a supernatant obtained by the cultivation of the isolated follicular tissue with GTH. Consequently, it is thought that GTH does not have the effect of inducing the egg maturation directly and that the secondary substance formed by the ovarian follicular tissue, which has now become the target organ by the action of GTH is the true egg maturation inducing hormone[1)-3)].

2.2 Isolating and Identifying the Hormones Inducing Egg Maturation in Red-spot Masu Trout

In 1985, Nagahama and Adachi isolated and identified an egg maturation inducing hormone for the first time in vertebrates using red-spot masu trout[5)]. In the first place, in order to obtain a rough estimate of the chemical character of the egg maturation inducing hormone of red-spot masu trout, cyanoketone, which is a biosynthesis inhibitor of the steroid hormones, was added to the ovarian follicle and GTH while they were being cultured together and this resulted in the complete suppression of the egg maturation inducing effect by GTH[6)]. On the basis of such results, the egg maturation inducing hormone of red-spot masu trout was assumed to be Δ^4-steroid, and the following procedures for the isolation and identification of egg maturation inducing hormones were performed. Using as the starting material the culture medium obtained by the cultivation of a large amount of large-size ovarian follicles of red-spot masu trout with the white salmon GTH, fractions

having the egg maturation inducing activity were refined from this culture medium. After the egg maturation inducing effect of the ether extract fraction was confirmed, it was partitioned by hexane-50% methanol in order to remove yolk and other impurities. Since the egg maturation inducing activity was observed only in the 50% methanol layer, this layer was then separated by the 60% methanol solvent by the high-speed (high-performance) liquid chromatography (HPLC) using a reverse phase partition column, and 20 fractions taken at 2-minute intervals were collected. Of these fractions collected, the <u>in vitro</u> egg maturation inducing activity was observed only in the tenth fraction (Figure 1). As a result of the analysis of this fraction using HPLC and thin-layer chromatography, it was revealed that a single substance was contained in this fraction and on the basis of the comparison with various synthetic steroids this substance was thought to be 17α,20β-dihydroxy-4-pregnan-3-one (abbreviated as 17α,20β-diOHprog). When it was further analyzed using a mass spectrometer, a peak corresponding to a molecular ion was observed at 332 as well as strong peaks at 287, 269, 229, 124, and 91, confirming that this substance was 17α,20β-diOHprog. On the basis of these findings, it was concluded that the true egg maturation inducing hormone formed by the action of GTH in the ovarian follicle just before the egg maturation period in red-spot masu trout is 17α,20β-diOHprog. In frogs, although a progesterone or a substance analogous to it was thought to be the egg

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maturation inducing hormone since earlier days, no definitive proof has yet been produced[7]. To this day, no reports have been produced concerning the intraovarian facilitating factors affecting the egg maturation induction in other vertebrates[8].

17α,20β-diOHprog is a kind of lutein hormone and it is formed by the reduction of the 20th oxo radical of its precursor 17α-hydroxyprogesterone by a steroid 20β-hydroxyl dehydrogenase (20β-HSD) (Figure 2). 17α,20β-diOHprog is found to have the greatest egg maturation inducing effect in many teleost including salmonids in <u>in vitro</u> situations[1],[9]~[11]. Of the ten or more varieties of steroids detected in red-spot masu trout, 17α,20β-diOHprog was also found to have the greatest egg maturation inducing effect and its minimum effective concentration was found to be 1-10 ng/ml[11]. 17α,20β-diOHprog was identified for the first time in 1960 by Idler et al. in the serum of sockeye salmon[12], and with the development of the radioimmunoassay in the 1980's the determination of its content in the blood became possible. Changes in the 17α,20β-diOHprog content in the blood accompanying sexual maturation have been examined in several species of teleost including red-spot masu trout, and these studies revealed that the 17α,20β-diOHprog levels in the blood of the salmonids are, without exception, low during the period of yolk formation and high during the period of egg maturation and ovulation[13]~[18]. With the dog salmon that swim up the Ishikari River in Hokkaido, the 17α,20β-diOHprog content in the

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blood was found to be very low 1 ng/ml from the time they are in the northern sea to immediately prior to the time of spawning and only in ovulating fish this level was found to be markedly increased (about 300 ng/ml)[19] (Figure 3). Consequently, it is highly possible that 17α,20β-diOHprog is the egg maturation inducing hormone common to salmonids. 113

2.3 Two-cell Type Model for the Formation of Hormones Inducing Egg Maturation

In fishes such as salmonids whose ovarian follicle development in the ovary is synchronous the level of estradiol-17β in the blood is generally high during the period of egg yolk formation[20]. However, as explained earlier, the 17α,20β-diOHprog level is high during the egg maturation period whereas the estradiol-17β level during this period is low. The examination of the steroid hormonal formation capacity of GTH in the ovarian follicle during these periods reveals that during the period of egg yolk formation estradiol-17β is formed in the ovarian follicle as a result of reaction with GTH, whereas during the egg maturation period 17α,20β-diOHprog is formed.

The ovarian follicular tissues of fishes are constructed, as in other vertebrates, with a thick basal membrane consisting of outer capsular cell layer and the interior granule membrane cell layer. In many species of fishes including salmonids, large cells having the morphological characteristics of steroid producing cells are

seen near the blood vessel of the capsular cell membrane[21]. Since the ovarian follicles of salmonids are large, it is possible to separate them into the capsular cell layer and the granule membrane cell layer with relative ease with the use of forceps under an anatomical microscope[22].

The use of these isolated cell samples made it possible to study the role of the capsular cell layer and the granule membrane layer with regard to the formation of the egg maturation inducing hormones[23)&24]. The capsular cell layer and the granule membrane layer, both of which were obtained from a large ovarian follicle that has completed the yolk formation and just before maturation, were cultured both separately and together with the GTH refined from dog salmon at 15°C for 18 hours and the 17α,20β-diOHprog and its precursor 17α-hydroxyprogesterone levels in the culture medium thus obtained were determined by the radioimmunoassay. Although no 17α,20β-diOHprog was formed when either of the capsular cell layer or the granule membrane layer was cultured with GTH, a large quantity of 17α,20β-diOHprog was formed when both layers were cultured together with GTH. On the other hand, 17α-hydroxyprogesterone was formed in a large quantity with the cultivation of the capsular cell layer by itself, and with the group in which both layers were cultured together, its level was found to increase during the initial 12 hours of the cultivation, with its level declining rapidly after that. However, with the cultivation of the granule membrane alone, no

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precursor was formed at all. On the basis of these findings, it is thought that both capsular cell and granule membrane layers are required for the formation of the egg maturation inducing hormone 17α,20β-diOHprog by GTH and that the precursor 17α-hydroxyprogesterone is formed in the capsular cell layer by the action of GTH and this precursor then moves to the granule membrane layer to be converted to 17α,20β-diOHprog by 20β-HSD 114
localized there (Figure 4). Recently, the presence of a high affinity GTH receptor which is specific to the white salmon GTH was revealed in the rough membrane fraction of the capsular cell and granule membrane layers of the egg maturation period.

The acceleration of the formation of the precursor 17α-hydroxyprogesterone by GTH in the capsular cell layer is through the adenylate cyclase cyclic AMP (cAMP) system. The cAMP acts on the cholesterol side chain cleavage enzyme to raise its activity. Although the facilitation of the 17α-hydroxyprogesterone formation in the capsular cell layer by the GTH is dependent on protein, this is not the case in the synthesis of RNA.

As explained earlier, since no steroid is formed when only the isolated granule membrane cell layer is cultured with GTH, it is surmised that, unlike with the case of the capsular cell layer, no enzyme to metabolize steroid is present in the granule membrane cell layer for the formation of the precursor. This is also apparent from the fact that the cultivation of the granule membrane cell layer with GTH and 17α-

hydroxyprogesterone results in the formation of a large amount of 17α,20β-diOHprog. However, in this instance, only a small amount of 17α,20β-diOHprog is formed if only its precursor is used without the addition of the GTH. These results demonstrate that the GTH acts directly on the granule membrane cell layer to raise the activity of 20β-HSD. This effect of 115
GTH to activate 20β-HSD can be substituted by dibutyl cAMP and folscholine*, which is the activator of adenylate cyclase, as well as by theophylline, which is the inhibitor of phosphodiesterase, and IBMX[25]. Moreover, the cultivation of the granule membrane cell layer with GTH results in detection of a large amount of cAMP in the culture medium. Therefore, it can be concluded that the adenylate cyclase-cAMP system plays a important role in the activation of 20β-HSD by the GTH in the granule membrane cell layer. Moreover, recently it became clear that the Ca-calmodulin system is involved in the activation of 20β-HSD induced by GTH, cAMP and folscholine.

Unlike in the case of the formation of the precursor in the capsular cell layer, the activation of 20β-HSD in the granule membrane cell layer is dependent on both the synthesis of protein as well as the synthesis of RNA[26]. In other words, the action of GTH, cAMP, folscholine and other substances to activate 20β-HSD is markedly inhibited by the RNA synthesis inhibitors such as actinomycin D, cordycepin and α-amanitine and by the protein synthesis inhibitors such as cyclohexamide

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* Translator's note: uncertain spelling.

and puromycin. When these findings are taken into consideration, along with the fact that at least 12 hours are required for the activation of 20β-HSD by the GTH in the granule membrane cell layer, it is presumed that GTH acts on the granule membrane cell layer to synthesize new RNA and through this it facilitates the synthesis of 20β-HSD itself. On the basis of results obtained so far, it is known that about 9 hours is required for this synthesis of RNA to take place. As can be seen here, it can be stated that the activation system of 20β-HSD by GTH in the granule membrane cell layer of salmonids is an excellent empirical system for researching the molecular mechanism of the cellular functional differentiation caused by the glycoprotein hormones. The results of the experiments obtained so far using this system strongly suggest that the gene activation mechanism is at work during the process of the GTH action. In order to advance this research, the refining of 20β-HSD from the post ovulation follicle of red-spot masu trout is being attempted.

2.4 Acting Mechanism of the Hormones Inducing Egg Maturation

The treatment of the goldfish ovarian follicle, which is in the state just prior to maturation but is sufficiently developed, with 17α,20β-diOHprog, which is externally applied, causes the germinal vesicle to decay and then the egg to mature several hours later. However, the administration of a minute amount of the same steroid into the interior of the egg by means of injection does not induce egg maturation. These

results suggest that the action site of 17α,20β-diOHprog is on the surface of the egg. A similar mechanisms is considered to be present with the inducing of egg maturation by progesterone in frog eggs and the presence of progesterone receptors on the egg surface as a result of bonding experiment in Xenopus was reported[27]. Consequently, the fact that the action site of 116
the egg maturation inducing steroid is located on the egg surface is thought to be a general characteristic of the vertebrates. Normally, steroid hormones are believed to enter the cell via the cellular membrane of the target cell to bond with either the cytoplasm or nuclear receptor to manifest its action, and this is in a marked contrast to the occurrence of the egg maturation inducing effect on the cell surface observed with progesterone and 17α,20β-diOHprog. Consequently this is of utmost interest in considering the action mechanism of the steroid hormones. In the case of frog eggs, the bonding of progesterone with the egg surface receptor causes a reduction in the activity of adenylate cyclase as well as a reduction in the amount of cAMP in the egg. That this is the trigger for a series of egg maturation processes is being considered. Although no experiment of this kind has been conducted with fish eggs, the addition of substances such as folscholine, which is the inhibitor of cAMP and phosphodiesterase, to the culture medium of goldfish also results in the egg maturation not taking place even when 17α,20β-diOHprog is added. This is of interest since this

suggests that in fishes, too, a reduction in the amount of cAMP acts as the trigger for the inducing of egg maturation. At present, no biochemical research has been conducted concerning the egg surface receptor for 17α,20β-diOHprog.

As explained here, egg maturation, instead of being induced by the direct action of the steroid hormone, takes place through the action of a substance, which is newly formed in the egg interior by this hormone. This substance is called a egg maturation accelerator, and dynamic research is in progress on the chemical properties and physiological actions of this substance using frogs and starfish[28]. Since the injection of a small amount of the cytoplasm extracted from the goldfish egg, which had matured as a result of a treatment with 17α,20β-diOHprog, into the immature egg of goldfish causes it to mature, it is thought that the egg maturation accelerator is also formed in the cytoplasm of mature goldfish eggs. Furthermore, the cytoplasm of this mature goldfish egg is capable of causing the immature starfish egg to mature also. This finding suggests that the biological activity of the egg maturation accelerator is widely interchangeable among animal species, supporting the results of the studies conducted so far using frogs and starfish.

It was recently noted that these egg maturation accelerators were mitogenic substances not only for meiosis but for the somatic cell divisions as well. 28) One is looking forward for an explanation of these factors themselves.

3. THE RELEASE OF SPERM

As explained before, in spite of the fact that the testes of the males of salmonids just prior to the spawning season are filled with morphologically perfect spermatozoa,

these spermatozoa cannot be squeezed out. Moreover, the 117
spermatozoa obtained from the testes at this time have no motility. On the other hand, during the spawning season, the milky white spermatozoa with motility can be readily obtained since the spermatozoa have already moved to the sperm duct. Since the dilution of spermatozoa contained in the sperm duct with a liquid such as distilled water causes them to start moving actively, the spermatozoa are thought to gain their motility during the stage of movement from the internal cavity of the testicular lobule to the sperm duct. This movement of the spermatozoa from the testicular lobule to the sperm duct in fish is called spermiation. In reality, the transfer of the spermatozoa to the sperm duct can be confirmed by applying pressure to the abdominal section. Physiologically speaking, the spermatozoa attain motility during this stage, consequently, this can be thought of as the final maturation of the spermatozoa. However, this differs fundamentally from the final maturation of the egg cell in the ovary in that the maturation division has been completed even in the spermatozoa which has not yet attained the state of spermiation and their nuclear phase is n. Although in the mammals and in certain species of amphibians, the separation of the spermatozoa from the Sertoli cells, in which their heads have been buried, is called spermiation, however, in the teleost the reproductive cells are never found with their heads buried in the Sertoli cells during the stage of spermatogenesis. With the male dog

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salmon found in the Sanriku area of Iwate Prefecture, the spermiation takes place suddenly when a large amount of spermatozoa are transferred from the testes to the sperm duct during a period of one half to one day. The spermatozoa attain motility simultaneously at this time[29]. As can be seen here, the spermiation in fishes, as with egg maturation, is a phenomenon that takes place in a very brief time and it is highly possible that the endocrine system is deeply involved with this process.

3.1 The Spermiation Inducing Action of 17α,20β-diOHprog

The activity of the androgen synthesizing enzyme system is heightened in the testes of salmonids during the period of spermatogenesis, resulting in the formation of testosterone and 11-ketotestosterone. Consequently, the blood levels of these hormones are high. However, when the spermiation period is reached after the completion of the spermatogenesis, the blood androgen level is decreased, and instead the 17α,20β-diOHprog level is suddenly increased[19),30),31)]. During this period, the activity of 20β-HSD in the testes is high whereas the activity of C_{20}-C_{17} side chain cleavage enzyme needed for the synthesis of androgen is low. The results of the 11-ketotestosterone and 17α,20β-diOHprog levels determined in the blood of male dog salmon during the time of migration from the north sea to the spawning ground are given in Figure 5. It was found that the 11-ketotestosterone level was high from the north sea to the spawning ground while high values for the

17α,20β-diOHprog level were obtained only with males that have arrived at the spawning ground and reached the stage of spermiation, as described earlier with the females[19]. Consequently, the possibility for the serious involvement of 17α,20β-diOHprog in the spermiation in the males of salmonids is suggested.

In order to examine the spermiation inducing effect of 17α,20β-diOHprog, an experiment to administer hormones was 118
attempted. Of the salmonids, the males of early maturing red-spot masu trout, which attain maturity in one full year and therefore are small in size, are most suited subject to be used in <u>in vivo</u> experiments to administer hormones. This experiment was conducted about one month prior to the spawning season, and none of the fish used had reached the stage of spermiation. As indicated by Table 1, the spermiation was induced in all of the subjects being injected with dog-salmon GTH or 17α,20β-diOHprog but the amount of spermatozoa released was greater with the GHT. No spermiation inducing effect was observed at all with testosterone or 11-ketotestosterone with the dose used (1 μg/g body weight)[32]. Such spermiation inducing action of 17α,20β-diOHprog is also observed in goldfish[33] and eels. In the experiment using red-spot masu trout, the level of 17α,20β-diOHprog in the blood after the administration of GTH was also examined at the same time and it was found to have risen as high as 50 to 80 ng/ml 119
one day after the administration of GTH. This high level of

17α,20β-diOHprog in the blood was maintained for the duration of the spermiation period. Therefore, it is highly possible that the spermiation inducing action of GTH is not direct but is through the facilitation of the formation of 17α,20β-diOHprog in the testes. As explained earlier, since the spermatozoa that have spermiated and moved to the sperm duct have gained motility, it is possible to consider that the motility was given to the spermatozoa as a result of the administration of 17α,20β-diOHprog. However, there is no empirical evidence to prove definitely that this attainment of motility by the spermatozoa is due to the direct action of 17α,20β-diOHprog. Rather, it seems more appropriate to consider that 17α,20β-diOHprog influences the motility of the spermatozoa indirectly by altering the moisture content, ionic composition or pH of the seminal plasma. In either case, the clarification of the precise mechanism of the spermiation inducing action of 17α,20β-diOHprog is hoped for.

3.2 The Mechanism of 17α,20β-diOHprog Formation in the Gonads

It was already stated that the formation capacity of 17α,20β-diOHprog in the testes is high during the spermiation period. The testes of this period are almost entirely occupied by the spermatozoa with the only other presence being a small amount of Leydig, Sertoli and other cells. The role of these cell groups comprising the testes in the formation of 17α,20β-diOHprog was examined using three kinds of tissues, testicular

slices, and testicular slices with the spermatozoa removed (including the Leydig and Sertoli cells) and spermatozoa[34] (Figure 6). The cultivation of a testicular slice with GTH or 17α-hydroxyprogesterone results in the facilitation of the 17α,20β-diOHprog formation. However, when spermatozoa are removed from this testicular slice, even the addition of these hormones does not result in the formation of 17α,20β-diOHprog. These results highly suggests the involvement of spermatozoa in the formation of 17α,20β-diOHprog in the testes. In actuality, a large quantity of 17α,20β-diOHprog is formed by the cultivation of spermatozoa with 17α-hydoxyprogesterone. Moreover, the analysis of the metabolites contained in the culture medium obtained by the cultivation of spermatozoa and ^{14}C labelled 17α-hydroxyprogesterone by thin-layer chromatography revealed that the main metabolite was obtained at the position corresponding to that of 17α,20β-diOHprog. The same results were obtained with the spermatozoa of eels. These findings indicate the localized presence of 20β-HSD in the spermatozoon and this was totally unexpected from the findings obtained with the ovarian follicular tissue. 120

4. CONCLUSION

By means of the efficient utilization of the advantages of the synchronous growth and maturation of the reproductive cells of the salmonids, the hormonal substances that are involved in the final maturation of the male and female gametes, as well as their formation and the mechanism of their

secretion, have been revealed gradually. Along with egg yolk formation accelerating hormone estradiol-17β, the identification, for the first time in the vertebrates, of the egg maturation inducing hormone 17α,20β-diOHprog in red-spot masu trout resulted in two varieties of the intraovarian effector that controls the growth and maturation of the egg in salmonids being determined. Both of these two varieties of steroid hormones are formed by the interaction (two-cell type model) of the capsular and granule membrane cells that are the constituent elements of the ovary as a result of the action of a glycoprotein GTH. These two varieties of hormones, instead of being formed simultaneously, are formed in a convenient manner, with estradiol-17β being formed during the egg yolk formation period and 17α,20β-diOHprog formed during the egg maturation period. This conversion in its function observed in the follicular tissue is induced by the GTH secreted immediately prior to the maturation of the egg and it seems that the gene activation mechanism is involved in this process. This is a particular field of research in which new developments are expected. However, the question of what precise mechanism is involved in the induction of egg maturation by 17α,20β-diOHprog is a problem to be resolved in future, since the true form of the egg surface receptor for 17α,20β-diOHprog needs to be clarified first. Due to the limited space available, the explanation concerning ovulation had to be omitted in this article. Although a little dated, an

excellent summary by Goetz[21] exists concerning the ovulation in fishes.

17α,20β-diOHprog also induces the final maturation of spermatozoa. The steroid formation in the testes also converts 121
from the androgen formation system immediately prior to the spermiation to the 17α,20β-diOHprog formation system. Although the mechanism of this conversion is not yet clear, the formation of the precursor steroid by GTH seems to play a key role. The formation of 17α,20β-diOHprog in the testes takes place as a result of an interaction between the spermatozoa and the somatic cells. Especially, the fact that 20β-HSD is localized in the spermatozoa is worthy of note in contemplating its physiological action. Concerning the hormone controlling mechanism of spermiation, it is at present only at a stage where the tip of a thread that will eventually lead to the analysis has finally been found through research using salmonids. There is a need to analyze in detail the total process of the spermiation induced by 17α,20β-diOHprog from the morphological, physiological and biochemical perspectives. The development of research on the female and male gametes formation using salmonoid fishes as subjects is greatly desired in future.

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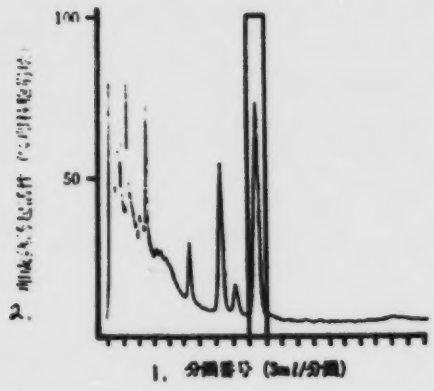

図1 エーテル抽出物の高速液体クロマトグラムと各分画の卵成熟誘起活性.

Figure 1: High-speed liquid chromatograph of ether-extracted substance and the egg maturation inducing activity of each fraction.

1. fraction number (3 ml/fraction)
2. egg maturation inducing activity (% of the germinal vesicle decayed)

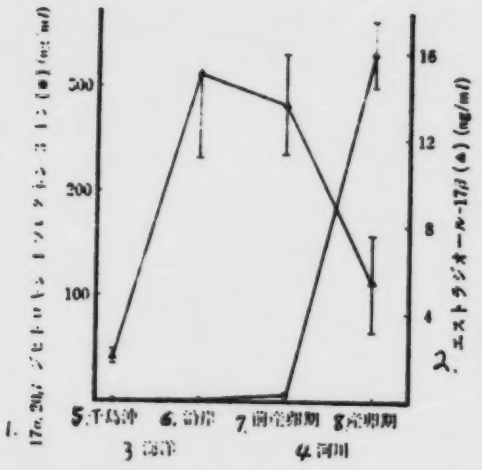

図3 シロサケ雌の産卵回遊時における血中 17α,20β-ジヒドロキシ-4-プレグネン-3-オンとエストラジオール-17β 量の変動.

Figure 3: The fluctuations in 17α,20β-dihydroxy-4-pregnen-3-one and estradiol-17β levels in the blood of dog salmon females during spawning migration.

1. 17α,20β-dihydroxy-4-pregnen-3-one (●) (ng/ml)
2. estradiol-17β (▲) (ng/ml)
3. ocean
4. river
5. off Chishima
6. coast
7. before spawning
8. spawning season

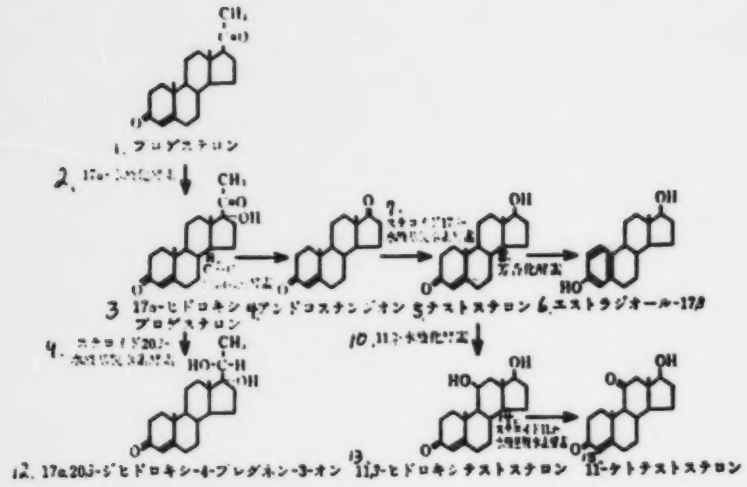

図 2 魚類生殖腺におけるステロイドホルモン生合成経路.

Figure 2: Steroid hormone synthesis pathway in the gonad of fishes.

1. progesterone
2. 17α-hydroxylase
3. 17α-hydroxyprogesterone
4. androstenedione
5. testosterone
6. estradiol-17β
7. steroid 17β-hydroxyl dehydrogenase
8. aromatization enzyme
9. steroid 20β-hydroxyl dehydrogenase
10. 11β-hydroxylase
11. C_{17}-C_{20} side chain cleavage enzyme
12. 17α,20β-dihydroxy-4-pregnen-3-one
13. 11β-hydroxytestosterone
14. steroid 11β-hydroxyl dehydrogenase
15. 11-keto testosterone

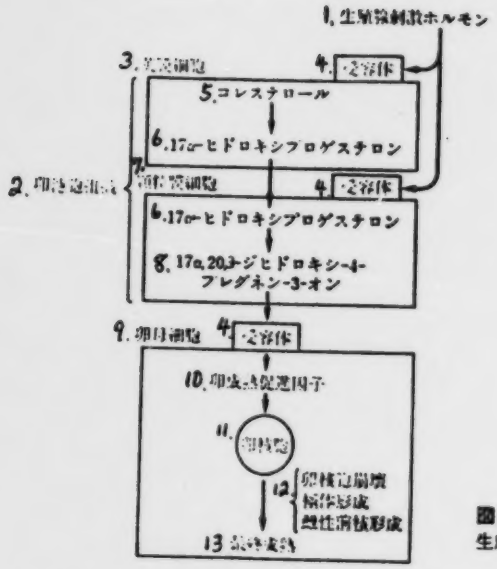

図 4 卵成熟誘起ホルモンの
生成・作用機構.

Figure 4: The formation and action mechanism of the egg maturation inducing hormone.

1. the gonadotropic hormone
2. follicle tissue
3. capsular cells
4. receptor
5. cholesterol
6. 17α-hydroxyprogesterone
7. granule membrane cells
8. 17α,20β-dihydroxy-4-pregnen-3-one
9. oocyte
10. egg maturation accelerator
11. germinal vesicle
12. germinal vesicle decay
    polar body formation
    female pronucleus
13. final maturation

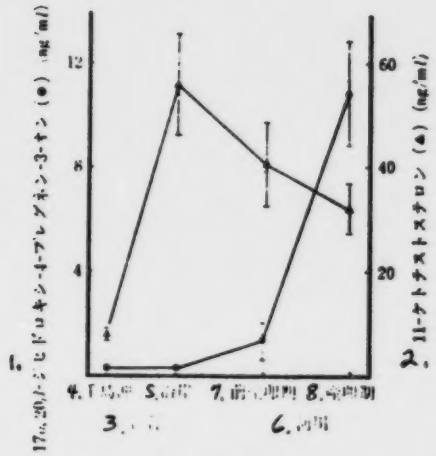

図5 シロサケ雄の産卵回遊時における血中 17α,20β-ジヒドロキシ-4-プレグネン-3-オンと 11-ケトテストステロン量の変動.

Figure 5: The fluctuations in 17α,20β-dihydroxy-4-pregnen-3-one and 11-ketotestosterone levels in the blood of male dog-salmon during the period of spawning migration.
1. 17α,20β-dihydroxy-4-pregnen-3-one (●) (ng/ml)
2. 11-ketotestosterone (▲) (ng/ml)
3. ocean
4. off Chishima
5. coast
6. river
7. prior to the spawning
8. spawning period

表1 ホルモン投与によるアマゴの排精誘起

1. ホルモン	2. 排精反応(尾数)		
	−	+	++
3. シロサケゴナドトロピン (SGA)			4
4. 17α,20β-ジヒドロキシ-4-プレグネン-3-オン		7	
5. 11-ケトテストステロン	7		
6. テストステロン	7		
7. 生理食塩水	6		

8. ホルモン投与量はいずれも 1 μg/g 体重. 投与回数はステロイドホルモンがいずれも2回で, SGA と生理食塩水は1回.

Table 1: Spermiation induced by the administration of hormone in red-spot masu trout.
1. hormones
2. spermiation reaction (number of fish)
3. white salmon gonadotropin
4. 17α,20β-dihydroxy-4-pregnen-3-one
5. 11-ketotestosterone
6. testosterone
7. physiological saline solution
8. The hormone dose was 1 μg/g body weight for all cases. The frequency of administration was twice for the steroid hormones and once each for SGA and physiological saline solution.

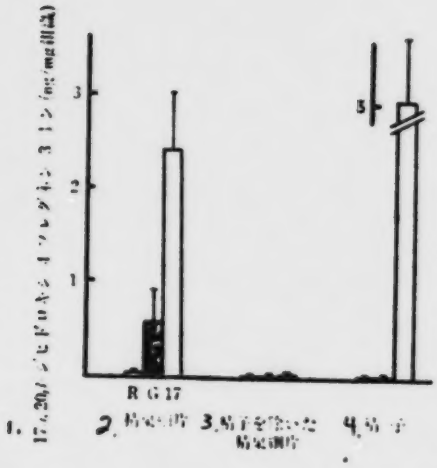

図 6 アマゴの精巣細片および精子における 17α,20β-ジヒドロキシ-4-プレグネン-3-オンの生成.
R：対照群（生理食塩水），G：シロサケ GTH(1 μg/ml)，17：17α-ヒドロキシプロゲステロン（100 ng/ml）

Figure 6: The formation of 17α,20β-dihydroxy-4-pregnen-3-one in the testicular slices and in spermatozoa in red-spot masu trout.

R: control group (physiological saline solution)
G: white salmon GTH (1 μg/ml)
17: 17α-hydroxyprogesterone (100 ng/ml)
1. 17α,20β-dihydroxy-4-pregnen-3-one (ng/mg tissue)
2. testicular slices
3. testicular slices with spermatozoa removed
4. spermatozoa

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9. Movement of the spermatozoa and fertilization in fish

(by Masaaki MORISAWA and Katsumi ISHIDA)

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3. Fertilization

The studies on the morphology, physiology and biochemistry of fertilization have yielded abundant results since the time when, more than 100 years ago, Hertwig observed under the microscope fertilization in sea urchins. The results are such that with regard to sea urchins whose research is the most advanced, detailed fertilization programmes have already been established[31]. At the present time, research on mammals is being actively pursued in the medicine and livestock fields. As for fish, one may say that up to the sixties, the studies on fertilization yielded for a period of time results exceeding certainly those obtained by the studies on other animals[32, 33]. As fertilization in fish takes place through passage via a particular structure called micropyle, even to the present day interesting research comprising electron microscope observation of fertilization is being pursued[34-37]. On a world scale however, physiological and biochemical studies in fish fertilization are few. We will content ourselves here to expose the basic knowledge about the fertilization in fish.

3.1 The penetration of the spermatozoa into the eggs

Cyclostomata such as lampreys perform fertilization outside the body. The eggs exhibit a peculiar shape. As shown in Fig. 4, there is transparent tuft at the animal pole, and the vegetal pole side is covered by a jelly layer. There is no micropyle as seen in cyprinodont eggs. The spermatozoa have a remarkable acrosome (Fig. 1). At fertilization time, the spermatozoa produce an acrosome reaction and penetrate through the animal pole[38-40].

All Elasmobranchii, sharks, rays undergo entosomatic fertilization. Some species however develop the fertilized eggs inside the uterus, while other species release the eggs outside the body so that the development takes place in the water. In any case, the spermatozoa penetrate the eggs inside the body, and the spermatozoa having produced the acrosome reaction go easily through the thin egg membrane. In these species, even in the case of normal fertilization, there are physiological polyspermic eggs which means that numerous spermatozoa penetrate one same egg. Yet, fertilization occurs when the female pronucleus fuses with the closest male pronucleus.

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Ganoidei, sturgeons perform fertilization outside the body. As Fig. 4 shows, the egg is coated with a thick egg membrane and has several micropyles at the animal pole. The spermatozoa have an acrosome[41] and penetrate through the micropyle. There are no polyspermic eggs as in the

case of sharks. With normal fertilization, the action is monospermic. The micropyle seems to have some antipolyspermic mechanism[42].

As explained earlier, many Teleosti are oviparous and undergo fertilization outside the body. Some species however are fertilized inside the body. The eggs are coated with a thick egg membrane and except for the eggs of some viviparous fish, have one micropyle (Fig. 4). The spermatozoa penetrate the egg through this narrow passage. The mode in which the spermatozoa approach and penetrate the eggs has been observed under optic microscope for a long time with regard to numerous species. Fig. 5 gives an example taken from herring[43].

Observations of insemination reveal that the spermatozoa removed far from the egg are virtually immobile. The motility of the spermatozoa having come close to the egg surface intensifies upon contact, and the spermatozoa move toward the micropyle. The spermic activation at the surface of the egg seems to be caused by the aforementioned spermatozoon-activating substances. The micropyle grows gradually narrower from the entrance on to the middle part, so that the spermatozoa line up in this same direction. It is said that these spermatozoa move their flagellum without being able to move forward. About 30 min following fertilization, those spermatozoa not involved in the fertilization process are suddenly ejected from the micropyle. Yet, at the next moment other spermatozoa enter the micropyle. The same phenomenon is repeated several times. The mode of fertilization by passage through the micropyle has been observed in the kurogashira (black-head) plaice, the top minnow and such. The

spermatozoa of these fish move actively from the start and penetrate the micropyle one after the other with gyrating motions. In the case of these fish, like for instance the plaice, the spermatozoa are expelled in the

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same way as with the herring. As the perivitelline space is formed, a spherical stopper closing the micropyle is also formed and penetration by spermatozoa is prevented[44].

3.2 Changes in the cortex

The egg protoplasm of the unfertilized fish eggs surrounds the yolk and is called cortex. Numerous oil globules and cortical alveoli with a diameter of about 10-14 μm are observed in the cortex. The cortex is surrounded in contact by the immediate cell membranes and the outside of the cell membrane is covered by a thick egg membrane. As the cell membrane and egg membrane are in close contact there is no perivitelline space (Fig. 5).

The spermatozoa go through the micropyle, or as in the case of the cyclostomata reach the cell membrane of the direct egg. When the mutual fusion of the egg and spermatozoon starts, a series of changes takes place in the egg. The first phenomenon is the collapse and disappearance of the cortical alveoli.

In 1936, Tchou and Chen conducted studies on the fertilization of goldfish. They observed that when the spermatozoa penetrate through the

micropyle at the animal pole, the contents of the cortical alveoli began to be expelled from the animal pole side, and that this change ended at

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the vegetal pole[46]. In 1941, Yamamoto researched this phenomenon in details, using top minnows, and proposed a "fertilization wave theory"[47]. Later, numerous reports on cortical changes were produced with regard to Cyclostomata, Ganoidei and Teleosti (see Bibliography 45).

The contents of the cortical alveoli of fish eggs consist mainly of mucopolysaccharides and of a small amount of protein. When the cortical alveoli collapse, the contents are expelled between the cell membrane and the egg membrane so that there is a production of colloidal osmotic pressure. The surrounding water enters the egg membrane, pushes the egg membrane upward and forms the perivitelline space. Simultaneously, the egg membrane hardens and grows stronger, and it becomes a fertilization membrane. In the course of the above cortical changes, the protoplasm gathers gradually at the animal pole and forms the scutellum. The spermatozoon changes inside the egg into the male pronucleus, fuses with the female pronucleus, and so is the fertilization completed (Fig. 6).

In this way, numerous observations of morphological changes comprising a few biochemical studies have been performed about the cortical changes in fish eggs. Yet, not very much is being revealed about the role played by these remarkable changes at fertilization time. Meanwhile, cortical changes are being examined in marine invertebrate

animals and more particularly so in sea urchins. These changes seem to be important in preventing polyspermism[31].

With exceptions such as sharks, generally only one spermatozoon

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penetrates the egg. This means that the mechanism is such that once the first spermatozoon has penetrated at fertilization time, no second spermatozoon will be able to get inside.

It seems that in sea urchins the polyspermic prevention mechanism works through two reactions, that is, one incomplete "fast" reaction occurring within a few seconds after the spermatozoon touches the egg, and a following "slow" reaction. The former fast reaction seems to proceed as follows. When the first spermatozoon touches the egg, there occurs within 1 sec an inflow of Na^+ ions inside the egg, which produces a temporary electric potential change in the cell membrane. This change in membrane potential constitutes the first obstacle to penetration by the next spermatozoon. It seems that the following slow reaction comprises changes of the egg cortex. In unfertilized sea urchin eggs too the immediate inner side of the cell membrane is called "cortex" as is the case of fish. Numerous granules (cortical granules) with a diameter of about 1 μm and smaller than in fish are present in this cortex. These cortical granules contain proteins, mucopolysaccharide sulfates, and enzymes. When fertilization takes place, the cortical granules fuse with the cell membrane, the contents of the cortical granules are expelled between the cell membrane and the egg membrane. Some of the contents combine with the

egg membrane and form a strong membrane, that is, the fertilization membrane. This membrane is said to constitute a wall preventing polyspermy.

In the case of fish, one observes membrane potential changes in fertilized eggs. One does not know with certainty whether the slow refusal of polyspermy in fish is due to the expulsion of spermatozoa in the micropyle, whether it is performed by the stopper, whether it is due to membrane hardening brought about by cortical alveolic matters or whether it is due to a joint action of all these factors. Likewise, it is unclear whether or not the membrane potential changes play a role in the rapid prevention of polyspermy. This will constitute an interesting topic for future research.

4. Conclusion

The salmonids were first in revealing us the beginning of motion and mobility acquisition in spermatozoa. With freshwater and seawater fish, osmotic pressure is the first trigger of motion start. If the mechanism became completely clear, further vistas might be opened for new studies not only with regard to the problems of fish multiplication but maybe with regard to the problem of cells in their totality also which is the response of the cells to pressure. We demonstrated recently that with the spermatozoa of the Xenopus, Rana and such other frogs the osmotic pressure was as in freshwater fish the factor of movement start in spermatozoa[48]. These findings indicate that even at the time when the organisms moved

from the rivers to land in the evolutionary process, the reaction mechanism did not change at spermatozoa releasing time if the fertilization environment was freshwater. If we also consider that when

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the animals moved from the sea to the rivers, the movement start factor of the spermatozoa changed from K^+ to osmotic pressure[19], then it becomes necessary to grasp the question of fish spermatozoa not as a question regarding fish only but as a question regarding all animals, and we believe that the question is quite important.

On the other hand, it does not appear for the present that through the study of fish we have explained the numerous mysteries concerning the eggs of the other animals. However, just like the spermatozoa, the eggs are supposed to have the fish characteristic of reacting to the environment, and studies in fish fertilization are concealing the possibility of bringing up new problems to be faced.

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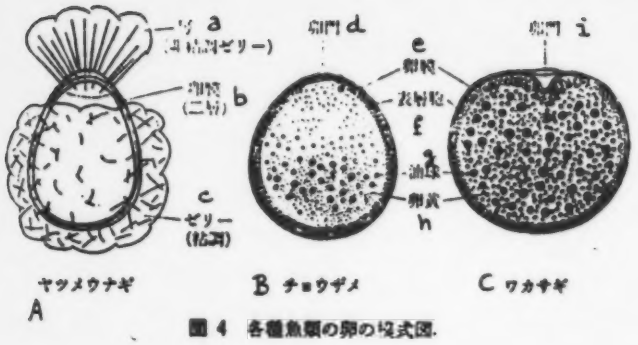

図 4 各種魚類の卵の模式図.

Fig. 4 Egg types of various fish species

A lamprey
B sturgeon
c pond smelt

a tuft (non crystalline jelly)
b egg membrane (2 layers
c jelly (viscous)
d micropyle
e egg membrane
f cortical alveoli
g oil globules
h yolk
i micropyle

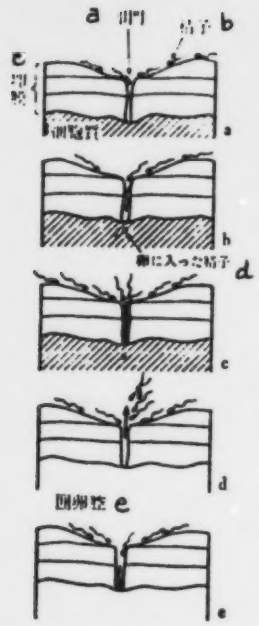

図 5　ニシン精子の卵門への侵入（柳町隆造，動物学雑誌，66 より改図）．a：媒精すると多数の精子が卵門付近で活発に運動を始める．b：まず 1 個の精子が卵門から卵表層原形質内へ入る．c：続いて 2 番目以後の多数の精子が卵門に入るが原形質内には入れない．d：過剰精子は囲卵腔が形成されたあとも卵門内に存在するが，媒精後 30 分ぐらいで突然卵門外へ吐き出される．e：つぎの瞬間，再び別の精子が多数卵門に入る．この吐出，侵入は数回繰り返されるが，最後にはすべての精子は卵門の外へ出される．

Fig. 5　Penetration of herring spermatozoa into the micropyle (YANAGIMACHI Ryuzo, Zoological Magazine revised graphs from 66).

a: at insemination time, numerous spermatozoa start moving actively near the micropyle. b: first one spermatozoon enters the cortical protoplasm from the micropyle. c: thereafter, a second and more spermatozoa enter the micropyle but are unable to penetrate the protoplasm. d: the excess spermatozoa are still inside the micropyle even after the perivitelline space has formed, but about 30 min after insemination, they are suddenly expelled. e: the next moment, other numerous spermatozoa enter again the micropyle. Expulsion and penetration take place several times. In the end, all spermatozoa are ejected from the micropyle.

a micropyle
b spermatozoon
c egg membrane
d spermatozoon having entered the egg
e perivitelline space

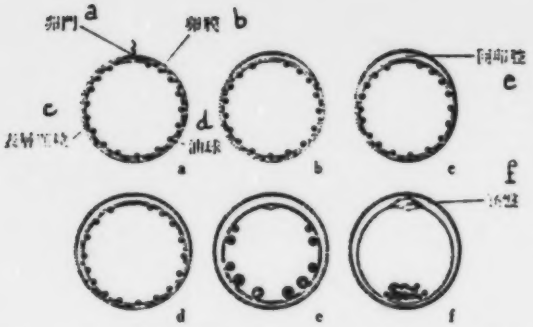

図 6　メダカ卵の受精過程（文献 32 より改図）．a：未受精卵．b，c：精子が卵門より入り卵と融合すると表層顆粒が崩壊し，囲卵腔ができ始める．d，e：表層変化が動物極から植物極へ伝播するに伴い，卵膜が細胞膜から離れ囲卵腔が広くなる．f：油滴は植物極に集まり，動物極に胚盤が形成される．

Fig. 6　**Fertilization process of top minnow egg (revised Fig. from Bibliography 32).**

a: unfertilized egg. b,c: when the spermatozoon enters from the micropyle and fuses with the egg, the cortical alveoli collapse and the perivitelline space begins to be formed. d,e: as the cortex changes are propagated from the animal pole to the vegetal pole, the egg membrane comes off the cell membrane, and the perivitelline space widens. f: the oil drops gather at the vegetal pole and the scutellum is formed at the animal pole.

a micropyle
b egg membrane
c cortical granules
d oil globules
e perivitelline space
f scutellum

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Translator's notes:

* reading probable but not entirely
** transliterated Japanese phonetic rendering of uncertain foreign name

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15. **Production of Growth Hormone by Genetic Recombinant Technology and its Applications** p.213

Akiyoshi TAKAHASHI and Koji KAWAUCHI

1. Introduction

Genetic recombinant technology has been established, which has made possible the large-scale production of peptide-type physiolocally-active micro substances, and they are used for the production of medicines such as human insulin, growth hormone (GH), interferon etc. If this technology is applied to the production of the GH of fish, it becomes possible to use the GH as a growth promoting agent in fish cultivation. As described in Chapter 2, the chum salmon (shiro sake) GH (sGH) and eel (unagi) GH have been identified, and the total structures of the genes (cDNA) of these fish GH have been determined. Furthermore, cDNA has been spliced in the plasmid of colon bacteria and GH production has been begun. Described in this Chapter will be the growth promoting effects on the salmon family of fish by the chum salmon GH (rsGH) produced by this method, and its application to promote fish cultivation.

2. Genetic Recombination of Chum Salmon Growth Hormone

The isolation and determination of the bases arrangements in the cDNA of sGH, the addition of this to the plasmid of colon bacteria, and the generation of chum salmon GH (rsGH) have been carried out by SEKINE *et al.*[1] as outlined below. First, the mRNA fraction from the chum salmon hypophysis was

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purified, and a cDNA library was prepared by the Okayama-Berg method. From this cDNA library, using synthetic DNA corresponding to the 23-28th positions of the amino acid arrangement of sGH, sGH cDNA was selected (Figure 1). From this gene, the section which codes sGH was excised with the restriction enzyme, and then the vector, which was excised by the restriction enzyme and which contains the triptophan promoter, was connected to the chemically synthesized DNA linker using DNA ligase, and by transforming to colon bacteria, the plasmid possessing sGH cDNA was prepared (Figure 2). This plasmid incorporates many innovative features, and the sGH content reaches 30% or greater of the colon bacteria fungal protein content. p.214

3. Growth Promoting Activity of rsGH

For estimating the effects on growth promotion by rsGH, rainbow trout fingerlings were used. The administration method was by abdominal injection, the amount of the feed ingested was as much as the fish would eat, and the results were compared that of sGH. As shown in Figure 3, the activities of rsGH and sGH were the same.

4. Administration Method

Attempts to promote the growth of fish by GH were begun in the 1930's. At that time the hypophysis extracts of mammals were used, but later mainly bovine GH was used, and its injection, insertion of cholesterol pellets or oral administration, etc. were studied. These research studies have been reviewed by Donaldson *et al.*[2] p.215

The growth promoting effects of sGH and rsGH have been established by the abdominal injection procedure, and so the application of rsGH to the promotion of fish cultivation depends on the development of a simple and effective administration procedure.

4.1 Immersion Treatment

Immersing the fish in a GH solution as a means to introduce the GH into the fish can be considered. This immersion method has been tested by Pickford[3] using bovine GH, but it was found to be not very effective. After that, Amend and Fender[4] showed that by using the immersion method, albumin is taken into the body of rainbow trout. Today this immersion method is used for the vaccination treatment of fish. Chum salmon and coho salmon were immersed in rsGH solution and the growth promoting effects were studied.

First, every 4 days, chum salmon fingerlings (15 in each group) were immersed in 3.5% brine for 2 minutes, and then in rsGH (30 ppm) solution for one hour. After 5 immersion cycles, up to the 24th day the daily body weight increases were 2 times that of the control group, and so a growth promoting effect was recognized. Next, for the coho salmon fingerlings (50 in each group), one time per week they were immersed for 60 minutes in 30 ppm and 3 ppm rsGH solutions, in 30 ppm bovine albumin solution, and in one containing no protein. The growth ratios of the GH immersion group are significantly higher than that of the albumin immersion group and the control group, while the growths of the 30 ppm group are greater than that of the 3 ppm group (Figure 4). Therefore, it can be said that this growth promoting effect is specific to GH. Although the mechanism for the introduction of GH is not yet clear, it has been reported that the gonadotropic hormone releasing hormone of the low molecular-weight peptides are rapidly taken into the blood from the gills of goldfish[5], and that when rainbow trout are immersed in a bovine serum albumin solution labelled with radio-isotopes, the radioactivity appears immediately in the gills and

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epidermis.[6] Therefore, the section where GH is taken in can be considered to be the gills.

4.2 Administration into the Stomach p.217

If the growth promoting effects could be obtained when GH is mixed into feed and orally administered, this would be the most practical, but because GH is a protein hormone, basic research is necessary in such areas as the problem of lost activity due to digestion and whether or not there is absorption from the intestinal wall, etc. Several results which suggest the absorption of protein from the intestinal wall have been reported. That is, it has been observed that when horseradish peroxidase is directly injected into the digestive tube of salmon-family fish fingerlings, it appears in the intestinal tissue, and as time passes, it is transferred to the basement membrane side.[7] On the other hand, in the blood of rainbow trout which had been fed fermented blood powder (?) made from cattle blood, the presence of antibodies against bovine serum albumin was also observed. The above findings suggest that there is an intake mechanism of protein from the intestinal walls. Thus, rsGH (200 μg) was administered into the stomach of rainbow trout (100 g) with a probe (sonde), and the GH level in the blood plasma was monitored by sGH radio immunoassay versus time (Figure 5). After 15 hours a rapid increase in the GH level was observed. This increase in the immuno radioactivity is considered to be due to the rsGH, which was administered into the stomach, being absorbed and appearing in the blood. In the future, whether this is intact GH or a fragment must be p.218 clarified, and whether or not growth is promoted by administering into the stomach must await future investigations.

5. Continuation of the Growth Promoting Effects

When a hormone is administered, the autogenous hormone secretion is inhibited due to the negative feedback mechanism, and it is feared that the cells which produce the corresponding hormone will atrophy. In the case where bovine GH was continuously injected into coho salmon, although growth was promoted during the administration period, it is said that the growth ratio reverted back to that of the control group when the administration was halted. However, as shown in Figure 6, when sGH is injected into the abdomens of salmon family fish, the daily growth ratios, compared according to body weight for the 24 days from the beginning of the injections, are 2.21% for the 1 μg/g injection group, 0.87% for the 0.1 μg/g injection group, and 0.32% for the control group. From the 24th day to the 110th day, the daily growth ratios were 5.1% for the 1 μg/g injection group, 5.9% for the 0.1 μg/g injection group, and 3.4% for the control group. With regard to growth after the injections ceased, although the dependency on the amount administered disappeared, the growth ratios of the sGH administered groups were larger than that of the control group. This phenomenon is also observed for chum salmon which had been administered rsGH by the immersion method. Also, even when sGH is injected just one time, growth promoting effects are observed, but a dependency on the amount administered is not observed (Figure 7). The GH concentration in the blood plasma of the rainbow trout is about 10 ng/ml.[8] If the total blood volume of a rainbow trout with a body weight of 10 g is assumed to be 10% of its body weight, the amounts of rsGH used in Figures 3 and 6 are 10-100 times the amount of autogenous GH in the blood. However, as shown in Figure 8, the rsGH administered into the abdomen is rapidly

decomposed. Therefore, the continuation of the growth promoting effect is not considered to be due to the administered GH remaining and functioning for several days. On the other hand, when GH is administered, it is known that the intake of feed increases.[9.10] Also for the series of experiments given above, for the GH administered groups, increases in the feed intake were observed. This is considered to be due either to the GH promoting the metabolism which brings about active feed intake or to it functioning on the appetite control of the central nervous system, and in the latter case, it is highly probable that, instead of the GH itself, a fragment reacts directly on the appetite controlling system. Thus, clarification of the continuation of the growth promoting effect is left for the future. mechanism

In this Chapter, in order to find a possible means of applying GH to fish cultivation, only the growth promoting action was described. In addition, sGH is also known to function to increase adaptability to sea water. Using fish GH which is produced by the application of genetic engineering, for migratory fish, in particular the salmon family, advances in basic research on silvering or their behavior in returning to the sea will be developed, and it is expected that study will be of some advantage to the fishing industry.

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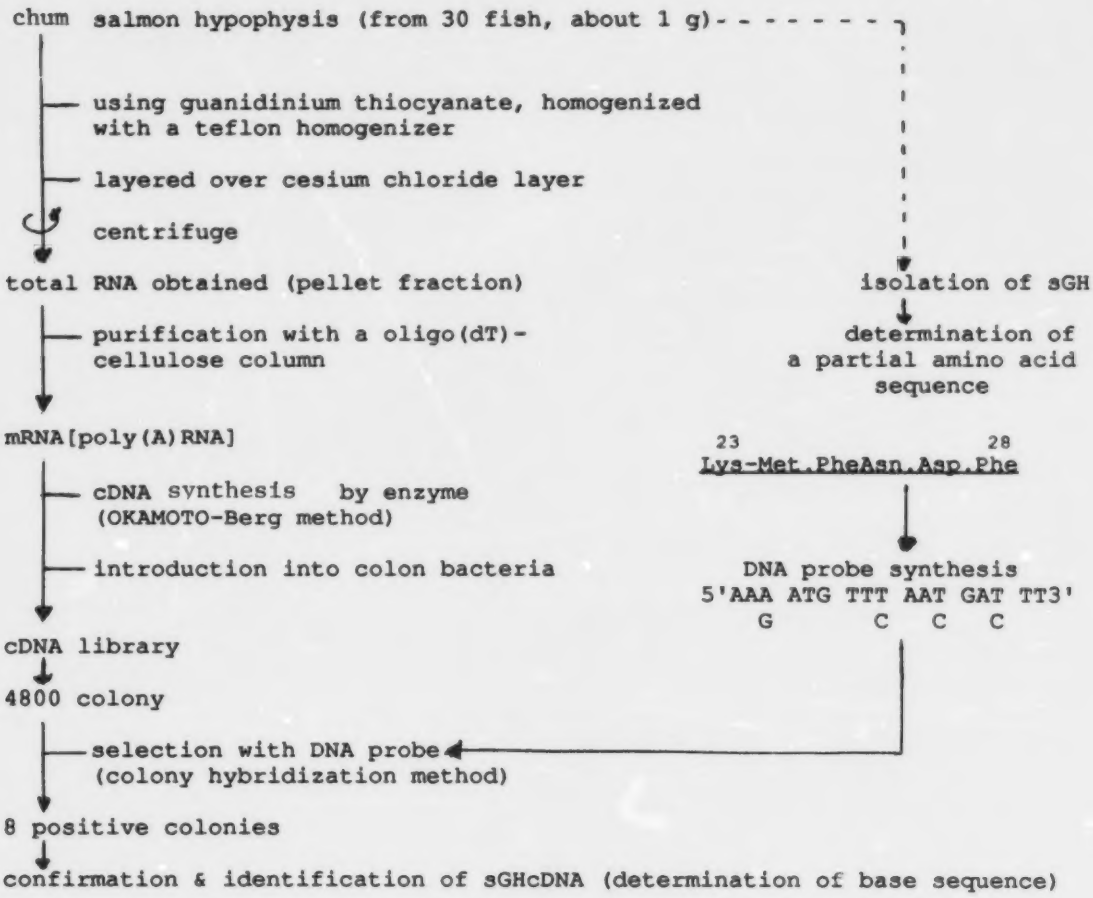
chum salmon hypophysis (from 30 fish, about 1 g)
using guanidinium thiocyanate, homogenized with a teflon homogenizer
layered over cesium chloride layer
centrifuge
total RNA obtained (pellet fraction)
purification with a oligo(dT)-cellulose column
mRNA[poly(A)RNA]
cDNA synthesis by enzyme (OKAMOTO-Berg method)
introduction into colon bacteria
cDNA library
4800 colony
selection with DNA probe (colony hybridization method)
8 positive colonies
confirmation & identification of sGHcDNA (determination of base sequence)
isolation of sGH
determination of a partial amino acid sequence
23 28
Lys-Met.PheAsn.Asp.Phe
DNA probe synthesis
5'AAA ATG TTT AAT GAT TT3'
G C C C

Figure 1. Method for Obtaining the chum Salmon Growth Hormone (sGH) gene (cDNA) from chum Salmon Hypophysis. DNA, which corresponds to the amino acid sequence (Lys^{23}·····Phe^{28}) of the section of the isolated and purified sGH, was synthesized, and then labelled with ^{32}P. The sGH cDNA is picked out using the property in which it specifically hybridizes with this DNA probe.[11]

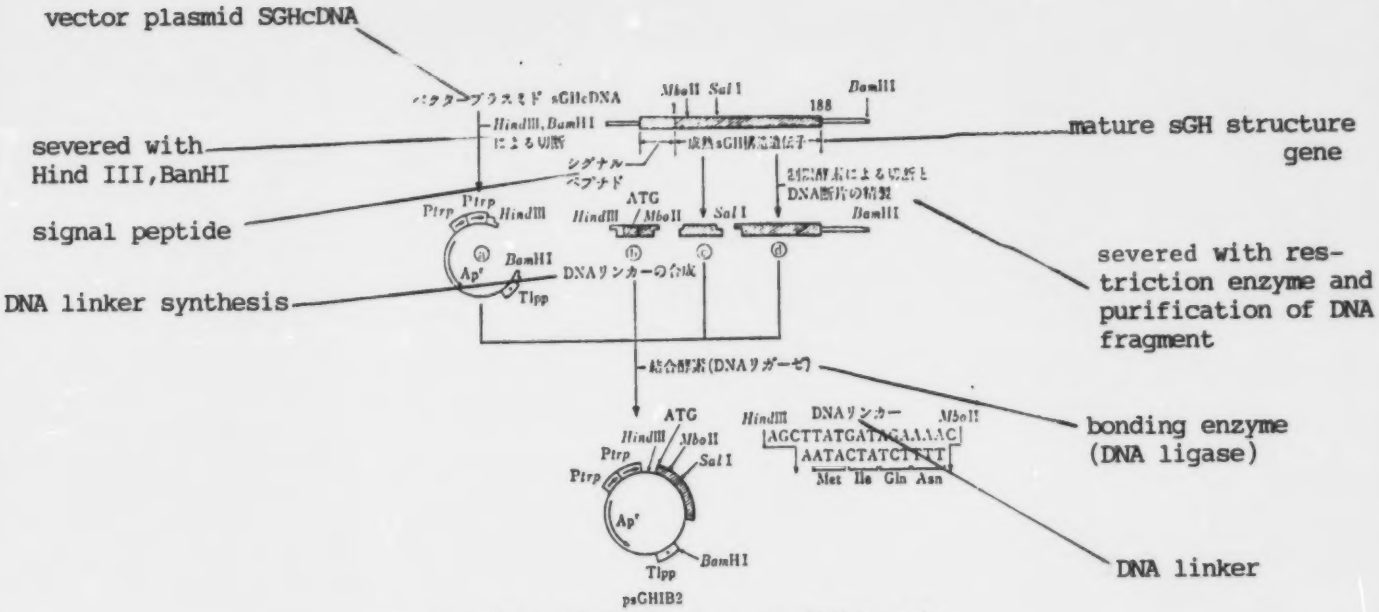

図 2 rsGH 生産用の組み換えプラスミド(psGHIB 2)の作製.
sGH cDNA から制限酵素により成熟 sGH をコードする部分を切り出し, DNA リンカー (ATG を含む) を介してベクタープラスミドに組み込む[11].

Figure 2. Preparation of Recombined Plasmid (psGHIB2) for the Production of rsGH.
From sGH cDNA, the section where mature sGH is coded is excised with the restriction enzyme, and with the DNA linker (including ATG) it is spliced into the vector plasmid.[11]

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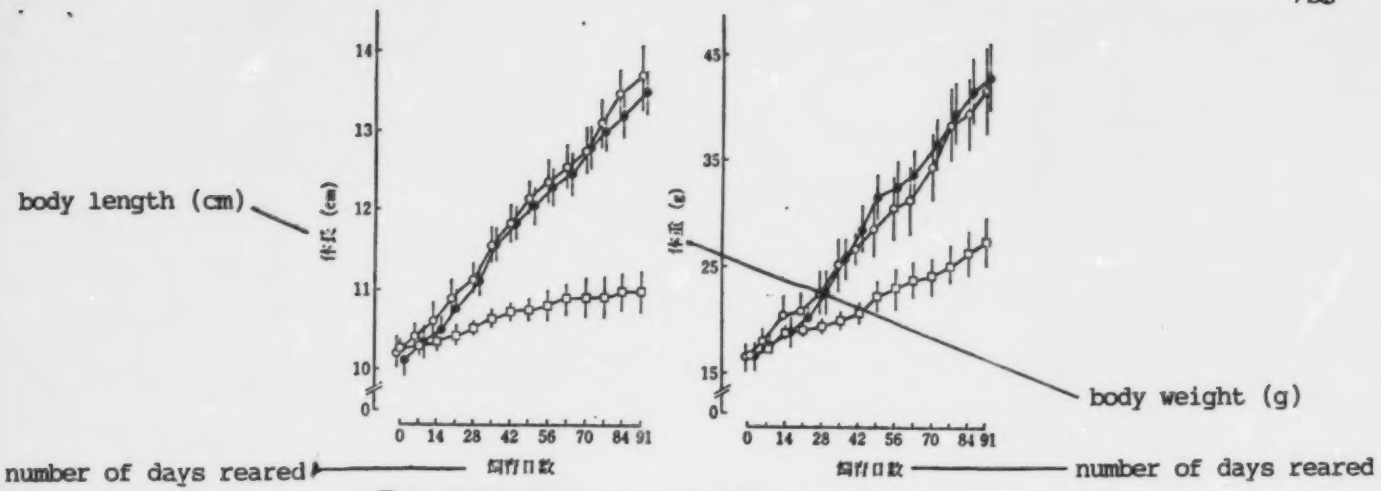

図 3 大腸菌の生産するシロサケ GH (rsGH) と天然のシロサケ GH (sGH) の成長促進効果の比較. rsGH 1 μg/尾 (●), および sGH 1 μg/尾 (○) を 0 日から 80 日まで 7 日ごとに 13 回ニジマスの腹腔内に注射した. □: 対照群.

Figure 3. Comparison of the Growth Promoting Effects of chum Salmon GH (rsGH) produced by Colon Bacteria and Natural Chum Salmon GH (sGH). rsGH 1 μg/fish (●) and sGH 1μg/fish (O) were injected every 7 days for a total of 13 times from day 0 to day 80 into the abdomen of rainbow trout. □: control group.

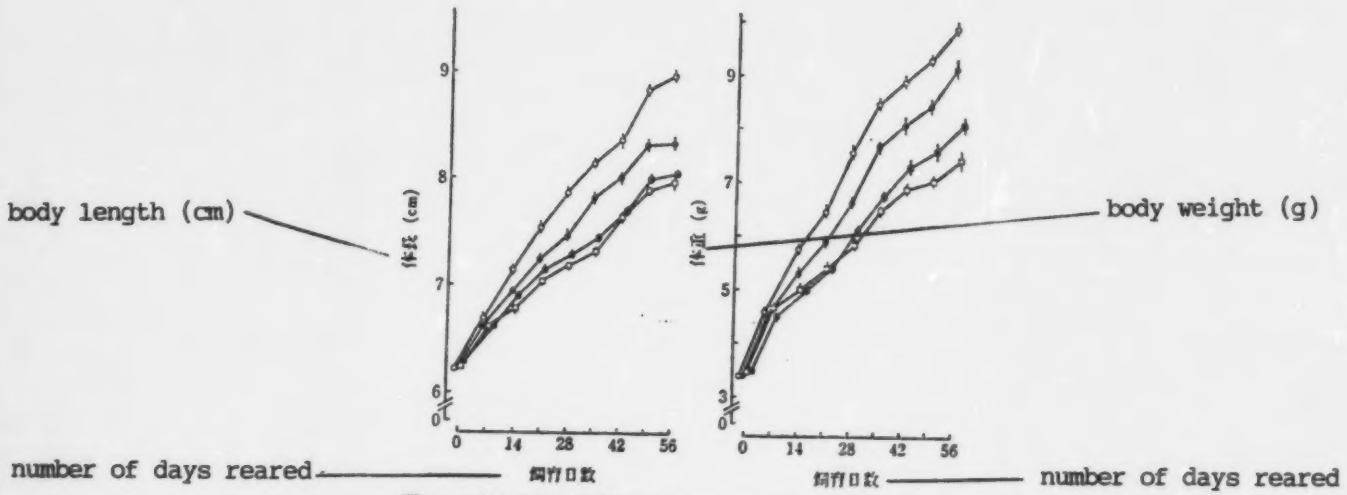

図 4 rsGH 溶液に浸漬処理したギンザケの成長. rsGH 溶液 (●: 3ppm, ○: 30ppm) および 30ppm ウシアルブミン溶液 (■) にギンザケを 60 分間, 7 日ごとに 8 回浸漬した. □: 対照群.

Figure 4. Growth of Coho Salmon Immersed in rsGH Solutions. Silver salmon were immersed for 60 minutes every 7th day for a total of 8 times in rsGH solution (●: 3 ppm, O: 30 ppm) and in 30 ppm bovine albumin solution (■). []: control group.

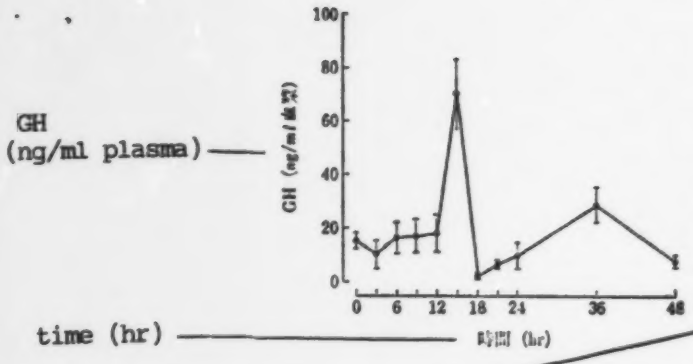

図 5 ニジマスに胃内投与後の血中 GH 量の変動. rsGH を胃内に投与後, 経時的に採血し血漿 GH 濃度をラジオイムノアッセイでモニターした (n=5).

Figure 5. Variation in the GH Amounts in the Blood after Administration into the Stomachs of Rainbow Trout. After rsGH was administered into the stomach, the plasma GH concentration was monitored with time using radio immunoassay (n=5).

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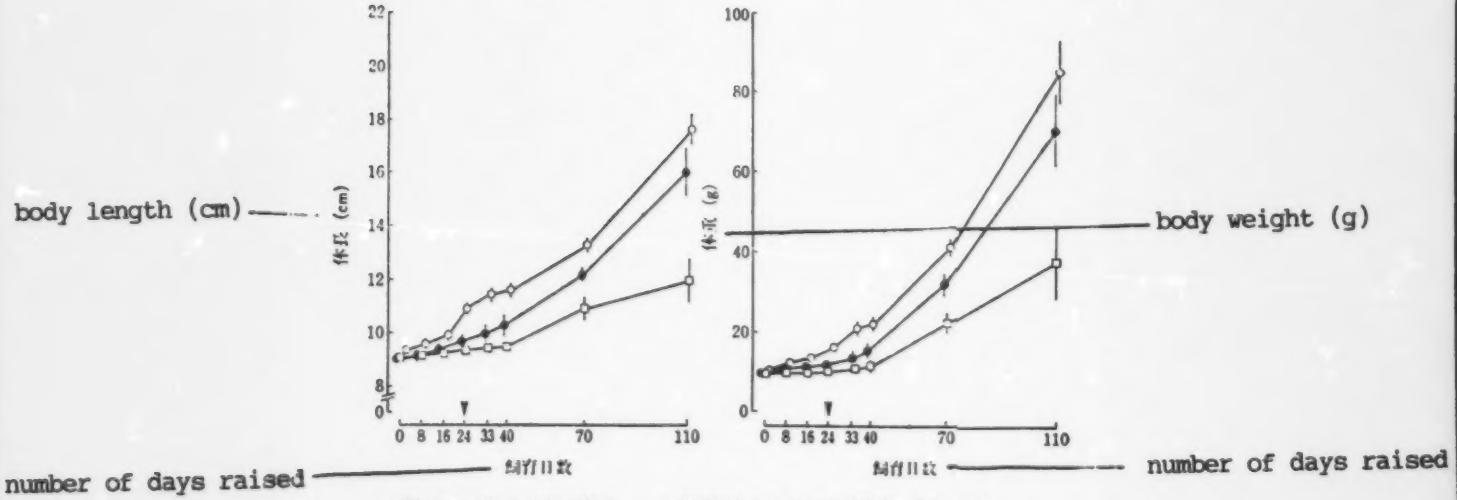

図 6 sGH の連続注射中, および注射後のニジマスの成長. 0.1 μg/g 体重 (●), および 1 μg/g 体重 (○) の sGH をニジマスに 0 日から 24 日まで 4 日ごとに 7 回腹腔内に注射し, さらに 110 日まで飼育を継続した. □: 対照群. ▼: 注射終了日.

Figure 6. Growth of Rainbow Trout during Continuing Injections of sGH and after Injections were Halted. From day 0 to day 24 at 4 day intervals, a total of 7 injections were administered to the abdomen of rainbow trout: sGH 0.1 μg/g body weight (●) and 1 μg/g body weight (○) of sGH; then rearing was continued until the 110th day. □: control group. ▼: day of last injection.

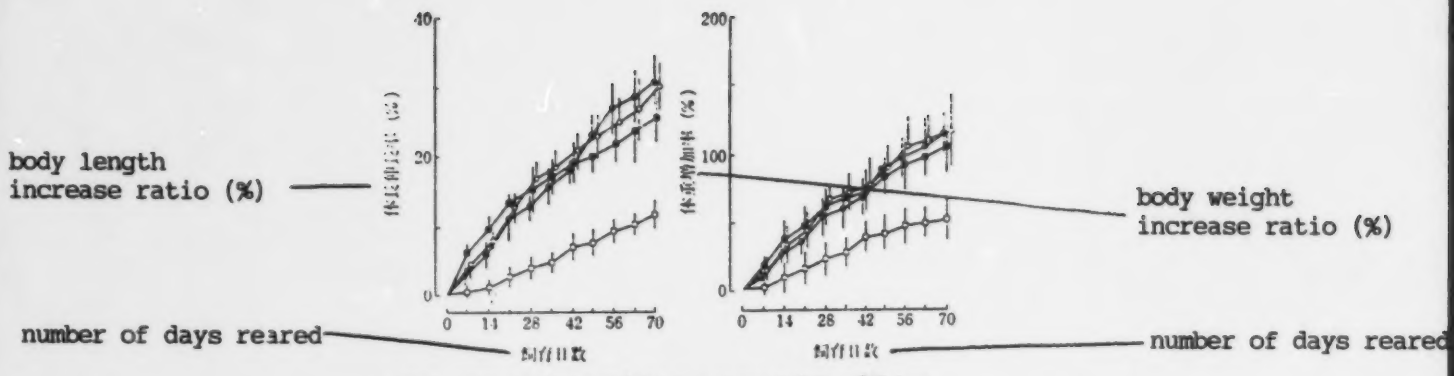

図 7 sGH を腹腔内に単投与したニジマスの成長. sGH 5 μg (●), 10 μg (○), および 50 μg (■) を 0 日に腹腔内注射し, 70 日まで飼育した. □: 対照群.

Figure 7. Growth of Rainbow Trout administered one time with sGH. sGH 5 μg (●), 10 μg (○) and 50 μg (■) were injected into the abdomen on day 0, and the fish were reared until day 70. □: control group.

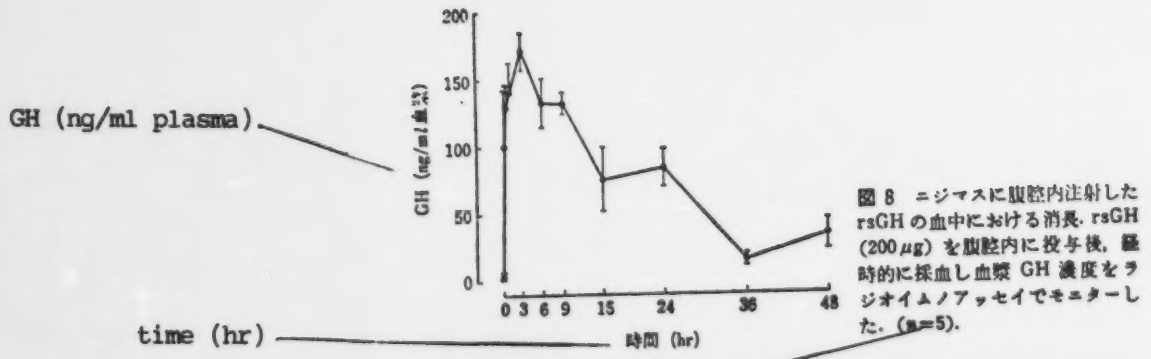

図 8 ニジマスに腹腔内注射した rsGH の血中における消長. rsGH (200 μg) を腹腔内に投与後, 経時的に採血し血漿 GH 濃度をラジオイムノアッセイでモニターした. (n=5).

Figure 8. Variations of rsGH in the Blood of Rainbow after Injection into the Abdomen. After rsGH (200 ug) was injected into the abdomen, blood sampled with time and the plasma GH concentration was monitored by radio immunoassay (n=5).

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16. Frozen Conservation of Fish Spermatozoa and Eggs p.222

H. KUROKURA

1. The Significance of the Conservation of Migratory Fish Spermatozoa and Eggs

It is very appealing to produce fingerlings of migratory fish and release them, and also to raise them so that their value is greater. However, on the down side, there are accompanying risks involved. For example, it is said that a female tuna fish spawns about 1 million eggs in one spawning period. From these eggs, if the released fingerlings are produced with a 20% recovery ratio, and if their return ratio is 2.5%, about 5,000 tuna fish spawned from one female will be recaptured. With the weight of one tuna calculated to be 0.2 t, 5,000 fish will be equal to 1,000 t, which is 1/36th of the approximately 36,000 t total weight of tuna caught in Japan in 1984. Now, if the fingerling production & release of tuna fish is successful commercially at the above described level, the result of the calculations is that almost all of the tuna groups which migrate to the area near the Japan coast would be the result of fish which are produced artifically from 36 parent fish. Under natural conditions, there are individual specimens with a variety of characteristics, and because of this, animals can preserve their species as a group by adapting

to the environmental changes. Therefore, when groups of fish are produced from a very small number of parent fish by artificial fertilization and made to adapt to a specific environment controlled by man during their fingerling stages, that is, groups without hereditary variations are created, they may be unable to adapt to the environmental changes and possibly die out. In other words, efforts to increase production may unintentionally deplete the hereditary resources which will be needed in the future. However, because of the above, if the biological resources are left alone they may become extinct, and so one cannot disavow the protection and propagation of these biological resources. If there is the possibility of disturbing the distribution of genes in nature, it is necessary to develop technology to return it to its original state, and for this, the conservation of the genes must be undertaken in the form of frozen conservation of spermatozoa and eggs. p.223

Aside from the necessity for this type of gene conservation technology, this technique is looked at with great expectation with regard to fish fingerling production sites, particularly from the aspect of salmon-trout propagation. This is so because with this technique it becomes possible to produce fingerlings independent of the natural maturing-spawning cycle. For example, the males of sweetfish seasonally mature more quickly than the female fish and then die of prostration. Because of this, by the time the females fully mature the number of males has decreased, resulting in a lack of spermatozoa.[v] It should be possible to solve this problem by preserving the sperm. Also, the conservation of fertilized eggs makes it possible to begin fingerling production under ideal conditions such as readiness of the feed animals, etc. Furthermore, carrying out frozen conservation of excellent quality fish spermatozoa, and

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using this technique in a much wider area to promote fish production, etc. are also important objectives of the conservation of spermatozoa and eggs.

2. The Current Situation for the Conservation of Fish Spermatozoa and Eggs

Research on the frozen conservation of fish spermatozoa began in 1953 when Blaxter[1] used frozen spermatozoa to cross the spring-spawning herring with the fall-spawning herring. This was one year after Polge and Rowson[2] succeeded for the first time in the frozen conservation of bovine spermatozoa, and the results were obtained (fish spermatozoa conservation) by almost directly using the research results on the frozen conservation of cattle spermatozoa. When past research results are examined, research on ocean fish or the fresh-water carp family of fish is very limited, with publication of research results very rare, but for ocean fish, like this herring case, a high level of fertilizing capability, similar to that of fresh spermatozoa, has been successfully preserved.[3,4] After that, research on the frozen conservation of fish spermatozoa has been mainly focussed on the salmon-trout family. The reason for this is that the method of producing fertilized eggs from the manually obtained eggs was known only for salmon and trout. However, the spermatozoa of salmon and trout were among the fish spermatozoa which were exceptionally difficult to preserve. It was in the later half of the 1970's when the fertilizing ratio of frozen spermatozoa of salmon and trout became comparable to that of fresh spermatozoa,[5,6] and this was brought about as a result of many years of research on the freezing rate and on the solvents for freezing. With a background history like this, at present it is considered to be possible to conserve or preserve the spermatozoa of almost any fish type,

and research is underway in the form of their application at actual sites to make these techniques more reliable and simple. p.224

Research on the preservation of fertilized eggs of vertebrates has been carried out mainly directed at small mammals. This type of research began in the 1950's when Ferdow[7] succeeded in obtaining offspring from the frozen embryos of rabbits. Encouraged by this success, many later follow-up research studies were conducted, but without success; and for research in this area to become active again, it was not until Wittingham[8] in 1971 succeeded in using the frozen fertilized eggs of mice. After that, the development of frozen fertilized-egg preservation techniques of mammals has been very rapid; in 1973 a successful case using bovine embryos was reported by Wilmut[9], and today, in the area of the livestock industry, the transplantation of frozen embryos is being looked at from the commercial side.

On the other hand, there has been very little research on the frozen preservation of fish eggs, and except for the research[10] on the tolerance of eggs to freezing for smelts, etc. which spawn eggs in the intertidal zone, etc., a very small number of fish such as rainbow trout,[11-14] coho salmon[13], salmon[14], herring[15], zebra fish[16], etc. became the object of frozen preservation research, but in general there has not been any success in the long-term preservation of fish eggs in the frozen state. This is considered to be so because fertilized fish eggs are larger than the embryos of mammals, etc. and also because they contain a large amount of egg yolk.

3. Frozen Conservation (preservation) of Fish Spermatozoa

The procedure for the frozen preservation of fish spermatozoa and its use for fertilization involves the 6 steps as shown below (Figure 1).

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These steps are 1) collection of the spermatozoa, and then 2) diluting them with an appropriate dispersing solution which contains the freeze-protecting substance. Then this is 3) frozen and 4) stored in liquid nitrogen. When required, it is 5) defrosted and 6) used to fertilize eggs. In each of these operations there are many aspects which require further study, and also the operation may have to be different for each fish type. When fish spermatozoa are classified according to the mobility of the sperms, they can be divided into 4 groups, which are the ocean fish, the salmon & trout type, the fresh-water fish other than salmon-trout, and the viviparous fish.[17] These differences are also significant with regard to the frozen preservation of fish spermatozoa. This is not a minor matter such as selecting the dispersing solvent for diluting spermatozoa. Here in this paper, with regard to the frozen preservation of the spermatozoa of ocean fish and the salmon-trout type of fish, technical aspects will be described such as the dispersing solvent (dilution) for diluting spermatozoa, the freezing rate (freezing method), and the defrosting-fertilizing method. With regard to much more technical information, there is a previously published review[18], and for the theoretical background on the frozen preservation of live cells, refer to the reference given.[19] p.225

The dispersed solvent for suspending the spermatozoa is prepared by mixing with the freeze-protecting substance in a diluting solution. The composition of this diluting solution has ordinarily been a very important item for investigation. This is because the movement of spermatozoa is triggered by changes in the osmotic pressure, ion composition, etc. of the environmental water, and moreover, for the salmon-trout type of fish, the time span of the motion is less than one minute, a very short period. That is, the diluting solution composition needs to be such that it suppresses

the movement of the spermatozoa to prevent a waste of energy. In order to achieve this, for ocean fish it must be a solution isotonic with saline solution, and for the salmon-trout type fish, in addition to these features, a necessary condition is that it contain K ions. In addition, a p.226
necessary condition is that the pH be weakly alkaline and stable. As solutions which satisfy these conditions, for the ocean fish there is sodium citrate solution, while for the salmon-trout type fish, dilution solutions with various compositions have been reported (Table 1). As the freeze-protecting substance, DMSO is often used. Especially for the salmon-trout type fish, except for the use of glycerine in some of the early cases, DMSO has been used in all the reports after that. A reason that can be given for this is that, in addition to the high level of DMSO's freeze-protecting effect, there is its speed of penetration to the cells. According to research[21] in which the equilibrium time (the time taken from mixing the spermatozoa with the dispersing solution to freezing) was p.227
investigated for the frozen preservation of tuna fish spermatozoa, when DMSO was the freeze-protecting substance, the equilibrium time was short, while when glycerine was used, the activity of the spermatozoa after defrosting remained high only when the equilibrium time was prolonged. Also, even for the spermatozoa of rainbow trout, when DMSO was used as the freeze-protecting substance, it has been reported[25] that a very short equilibrium time is necessary. The spermatozoa of salmon-trout are susceptible to dilution shock, and the degree of damage increases along with the equilibrium time. Even from this aspect, a freeze-protecting substance such as DMSO, which penetrates rapidly, is suitable for the salmon-trout type spermatozoa. The next important factor is the freezing rate, but there are only a few comparison studies specifically on the

freezing rate. The present situation is that several freezing methods have been compared, and from these results an optimum freezing rate has been estimated. KUROKURA *et al.*[20] have carried out investigations with rates between -20°C/minute and -150°C/minute on the optimum freezing rate for crimson sea bream, but there were no differences, with respect to the freezing rate, in the spermatozoa activity after defrosting. On the other hand, KAWASHIMA *et al.*[26] reported on a method in which the spermatozoa were sealed in a straw tube and placed in alcohol cooled to various temperatures to study the freezing rate of rainbow trout spermatozoa; and the results showed that when immersed in -40°C alcohol, the fertilizing capability of the spermatozoa was maintained the best. In p.228 this experiment a type of two-stage freezing method was employed where after the spermatozoa reached the temperature of the alcohol, they were transferred to liquid nitrogen for storage. Therefore, factors other than the freezing rate itself, such as the alcohol temperature, the dilution at that temperature, etc. can be considered to have an effect. KAWASHIMA and NAKAGAWA[27] have reported that the freezing rate of spermatozoa which were soaked in -40°C and -50°C alcohol were -38.7°C/minute and -48.4°C/minute, respectively (Figure 2). These values are similar to the freezing rate for the pellet freezing method (the freezing method in which the spermatozoa are dropped onto dry ice to freeze them), which is used often today for salmon-trout type spermatozoa. However, the temperature of dry ice is -78°C, which is quite different from the alcohol temperature of -40°C in KAWASHIMA's experiments. From the above results it may not be wrong to surmise that in KAWASHIMA's experiments the important factor for maintaining the fertilizing capability of the spermatozoa is the freezing rate. That is, even with regard to the freezing rate, the salmon-trout

type fish have different characteristics than those of the ocean fish, etc. Thirdly, an often overlooked point is that the defrosting and fertilizing methods of spermatozoa are also important factors if the frozen spermatozoa are to be used in a practical way. As previously clarified, the movement of spermatozoa for the salmon-trout type fish is triggered by decreasing the potassium ions in the environmental water, and for the fresh water fish of the carp family it is triggered by lowering the osmotic pressure.[17] However, the frozen spermatozoa of rainbow trout and carp, etc. begin moving immediately after being defrosted, even without the above triggering conditions. It is known that when live cells are frozen, the osmotic nature of the cell membrane changes and substances in the cells leach out or substances are taken into the cells. KUROKURA and HIRANO[28] reported that when the spermatozoa of rainbow trout are frozen and defrosted, the K ions leach out of the cells and the Ca ions are taken into the cells. This movement of ions is very similar to the initial changes when the spermatozoa of the salmon-trout type fish begin to move.[29] Of course, by simply giving these ion movements as the cause of the initial motion at the time of freezing and defrosting, means there are still more investigations to be conducted in the future, such as the mechanism of ion intake, the process of the start of the movements, etc. However, for ocean-fish spermatozoa, a similar movement of ions can be observed by freezing and defrosting, but the spermatozoa do not begin moving. This is probably due to the other factors for initiating the movement of ocean fish spermatozoa being different from those of the salmon-trout fish type. This supports the previously given assumption or argument. In any event, the frozen spermatozoa of salmon-trout begin moving after defrosting and the time

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period of the movements is short, so that the results of fertilization with frozen spermatozoa are unstable. For rainbow trout it has been reported that the fertilizing capability decreases 30 seconds after defrosting.[30] p.229 Compared to fresh spermatozoa, it cannot be denied that the fertilizing capability of frozen spermatozoa is lower. In order to overcome this deficiency, the fertilization must be done immediately after defrosting, and also, it is important to fertilize under the optimum conditions suited for each fish type.

4. Frozen Preservation of Fertilized Fish Eggs

There has been very little research on the frozen preservation of fish eggs; there is a case in which the fertilized eggs of Atlantic salmon were cooled to -55°C, which is perhaps the lowest temperature at which fish eggs have survived,[11] but even for this case it was a short period of time that the eggs were kept at -55°C, and so there is the question of whether the eggs actually cooled down to that temperature. For all other fish types that have been studied, such as rainbow trout,[11-14] herring,[15] zebra danio (zebra fish, *Brachydanio rerio*),[16] etc. there has been no success in keeping fish eggs alive at -20°C to -30°C and lower. Among these studies, the research conducted by Harvey[16] using zebra danio is noteworthy because it showed some possibilities for the future. In his experiments, along with freezing the intact fertilized eggs, the freezing and thawing were tested on blastoderms with the egg membranes and yolks removed, on individual cells, etc. As a result, it was found that the intact fertilized eggs perished when cooled to -25°C, but the embryos survived at -25°C, while the individual cells survived even in liquid nitrogen at -196°C. This indicates that the low tolerance to freezing of the fertilized fish eggs is not due to the low tolerance of the individual

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cells, but is due to the following: the covering is a relatively thick egg membrane, the egg yolk is inhibiting freezing, the embryo is quite large as a whole, etc. Among these factors, the presence of the egg membrane inhibits the intake of the freeze-protecting substance (cryoprotectant) into the embryo as well as inhibiting the dehydration from the embryo, etc., while the presence of the egg yolk lowers the tolerance to freezing of the egg by making the egg larger, by making its physical nature non-uniform, etc. If these problems can be solved technically, the frozen preservation of fish eggs should become possible. Stoss and Donaldson[14] carried out experiments by fertilizing the eggs in an isotonic salt solution, and then freezing them without having them come in contact with water. This was done to prevent the egg membranes from hardening due to contact with water and to prevent the decrease in permeability of water; this is one of the useful methods which compensates for the shortcoming in fish eggs.

Although it is important for the preservation of fertilized eggs, the problems associated with the developmental stages of the egg have not yet been investigated. Not only to freezing, but to temperature, vibration, light, etc., the tolerance of eggs to these stimuli varies with the p.230
(Halichoeres poecilopterus - Temminck & Schlegel)
developmental stage. Figure 3 shows the hatching ratios of Kyusen fertilized eggs which developed at 27°C, where at each developmental stage they were soaked in sea water at 4°C for 30 minutes. For eggs which were cooled during the period between the 2-cell and morula stages, which includes the cleavage stage, the early gastrula stage, the embryo-forming stage and the blastopore closing stage, the hatching ratios decreased. Figure 4 is an example of an egg which was subjected to low temperature p.231

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during the cleavage stage. The size and shape of the cleaved eggs are not uniform, and this clearly indicates that the cleaving process is abnormal. During the cleavage stage, cell division is quite active, and subjecting to low temperature inhibits mitosis. As a result, the abnormalities must have occurred in the cleaving process. Figures 5 and 6 are examples of eggs which were subjected to low temperature during the early gastrula stage. The embryo of Figure 5 has two tails. In the embryo of Figure 6 development as a whole has progressed with segmentation occurring, but the blastopore is not closed. The major changes in the embryo which begin during the gastrula period are that, along with the growth of the periblast, the bastodisc covers the egg yolk, the deep cells of the blastodisc gather toward the back to form an embryo, and differentiation of each of the organs begins. The Figure 5 example is the result in which the movement of the cells was delayed, and before the cells could gather toward the back, differentiation of the tail section occurred, and the Figure 6 example is the result where the growth of the periblast was delayed and segmentation occurred before the blastopore was closed. In any event, the application of stimuli during the early gastrula stage is considered to inhibit the cell movements such as the gathering of the deep cells to the back, the growth of the periblast, etc. The eggs subjected to low-temperature stimulation during the embryo-forming stage and the blastopore closing stage perish immediately after stimulation and the eggs are physically broken down. From this, the eggs during these stages are considered to be structurally weak. The tolerance to stimuli including low temperature increases after the blastopore closes. Table 2 shows the results of freezing and cooling to -20°C the fertilized eggs of

flounder. The eggs which were frozen before the blastospore closing stage (before the formation of the eyes) did not hatch at all, but after this blastopore closing stage, the hatching ratios increased rapidly. After the p.232 blastopore closing stage stage, the frequency of cell divisions is low, and the cell movements also decrease. Also, structurally, the egg yolk is covered by the blastodisc and it is stable. Furtheremore, the size of an individual cell is smaller, and so the tolerance to freezing should be greater. In this experiment, most of the small fish which hatched from the frozen eggs were deformed. When freezing is carried out, one cannot expect that no damage will occur on the entire embryo. For the embryo in the later stages of development, the organs have differentiated, and so the damaged cells cannot be replaced by cells from other sections. Therefore, if these are preserved by freezing, it is quite clear that deformations will occur. However, these results provide some information for future research. That is, similar to the post blastopore-closing stage, the later blastula stage has a high tolerance to the stimuli, and moreover, the fact that the organs have not yet differentiated might mean development could be normal following frozen preservation. Also, if due structurally to the egg yolk being covered by the blastodisc, the tolerance to freezing of the embryo in the post blastopore-closing stage is high, then the unfertilized egg in which the egg yolk is surrounded by cortico-protoplasm may be simpler to freeze than the fertilized egg. There has not yet been any research done which focuses on these possibilities. Also, there are no detailed investigations with regard to the freezing rate, the solutions for freezing, etc. Thus, the frozen preservation of fish eggs is still at a stage where the possibilities are being examined, and in the future, by

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investigating from various perspectives, the frozen preservation of fish eggs may become possible.

5. **The Future of the Preservation of Fish Spermatozoa and Eggs** p.233

The preservation of fish spermatozoa is already in practical use, but the preservation of eggs is still in its infancy with regard to research, and its possibilities cannot be stated at this stage. However, even assuming that is not possible to preserve eggs, if for example the following procedures could be developed, the consequences would be similar in result to bringing about the successful preservation of eggs: using artificial male-developing techniques for promoting egg development with only genes from frozen spermatozoa; preserving the oocyte or oogonium and then maturing them; preserving blastodiscs by freezing, and after thawing let them develop normally; etc. When predicting the future in this manner, one notices that there is a major flaw in our research. The research so far has all been concerned with genetic preservation methods, but the type of gene that should be preserved has never been considered. If one does not examine the heredity of various characteristics of the migratory fish which are the objects of the propagation and decide the genetic features to be preserved, the eggs and spermatozoa will be preserved randomly, and the practical applicability of the genes thus preserved would be of questionable value. In the future, genetic and breeding studies on migratory fish will also become important. Also, supported by these kinds of results, practical application for research on spermatozoa and egg preservation will be brought about.

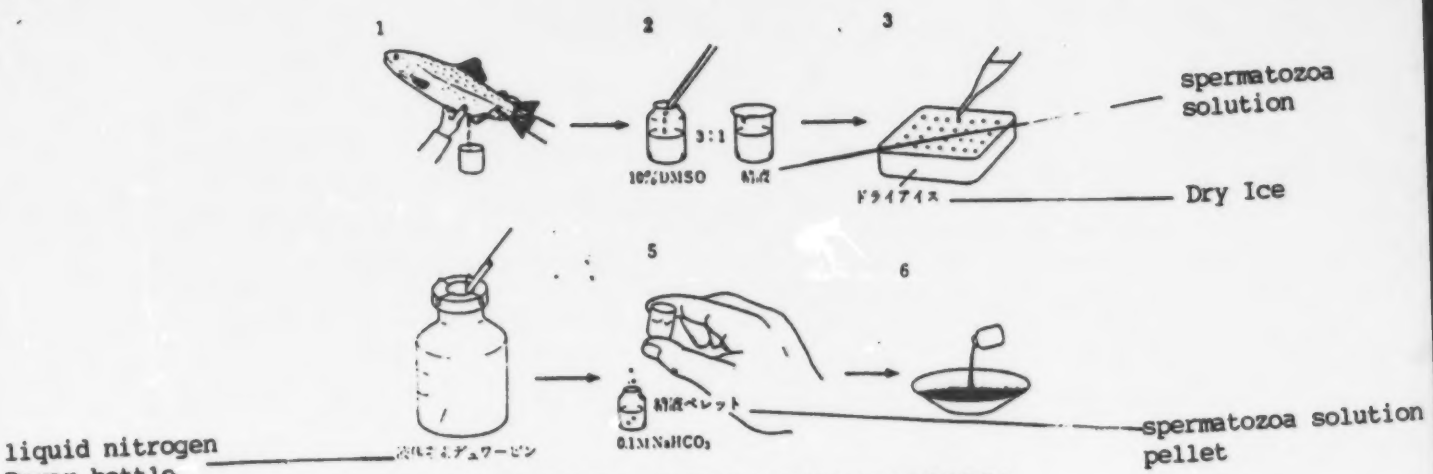

図1 魚類精液の凍結保存の一例（広島大学生物生産学部水産増殖学教室で行なっているニジマス精液の錠剤化凍結法）．（1）ニジマスの雄成魚の体表をよくふき，尿・環境水など異物の混入に注意しながら，搾出法により精液を採取する．（2）DMSO と希釈液（Stoss の希釈液を一部改変）を 1：9 の割合で混合する．この溶液3容に対し精液1容を加える．（3）混合後，すばやく，駒込ピペットを用いて，ドライアイス上の穴に希釈精液を滴下し，ペレット状の塊とする．1つのペレットの大きさは 0.1～0.3 ml．（4）凍結した精液を適当な容器に入れて液体窒素中に保存する．（5）凍結精液が必要な際には，0.1M $NaHCO_3$ 液 10ml に，ペレット状の精液3個を液体窒素からとり出してただちに投入し，素早く混合して精子懸濁液をつくる．（6）卵 20g にこの精子懸濁液を加え媒精する．

Figure 1. An Example of the Frozen Preservation of Fish Spermatozoa (Pellet-type freezing method for rainbow trout spermatozoa carried out by the Aquatic Propagation Group, Biological Production Department, Hiroshima University). (1) Thoroughly wipe the body surface of the male rainbow trout, and then avoiding contamination by urine and the environmental water, the spermatozoa are obtained by squeezing. (2) The DMSO and the diluting solution (partial modification of the diluting solution of Stoss) are mixed in a 1:9 ratio. To 3 parts of the resulting solution, 1 part of the spermatozoa is added. (3) After mixing, the diluted spermatozoa solution is immediately dropped with a Komagome pipet into the holes in the dry ice to form pellet-type solids. The size of one pellet is 0.1-0.3 ml. (4) The prepared spermatozoa are placed in a container and stored in liquid nitrogen. (5) When the frozen spermatozoa are required, 3 pellets are removed from the liquid nitrogen and quickly added to a 10 ml of 0.1M $NaHCO_3$ solution, and stirred immediately to make a spermatozoa suspension. (6) For fertilization, this spermatozoa suspension is added to 20 g of eggs.

Table 1. The Major Diluting Solutions used for the Frozen Preservation of Fish Spermatozoa

表 1 魚類精液の凍結保存に用いられるおもな希釈液

海産魚一般		サケ科魚一般	
〈クエン酸ナトリウム液[20]〉		〈Mounib の希釈液[5]〉	
クエン酸ナトリウム（二水和物）	2,940mg	$NaHCO_3$	840mg
ブドウ糖		スクロース	4,279mg
〈グルコース液[21]〉		グルタチオン（還元型）	200mg
グルコース	10,000mg	〈Stein の希釈液（V 2）[6]〉	
ボラ		NaCl	750mg
〈海産魚用リンゲル液[22]〉		$NaHCO_3$	200mg
NaCl	1,350mg	Na_2HPO_4	53mg
KCl	60mg	$MgSO_4 \cdot 7H_2O$	23mg
$CaCl_2$	25mg	KCl	38mg
$MgCl_2$	35mg	$CaCl_2 \cdot 2H_2O$	46mg
$NaHCO_3$	2mg	グルコース	100mg
コイ		グリシン	500mg
〈淡水魚用リンゲル液[23]〉		（この溶液 100ml に対してニワトリの卵黄 20ml を加える）	
NaCl	750mg	〈Stoss の希釈液[24]〉	
KCl	20mg	NaCl	592mg
$CaCl_2$	20mg	KCl	172mg
$NaHCO_3$	20mg	$CaCl_2$	68mg
〈コイ用人工精漿[23]〉		$MgSO_4$	15mg
NaCl	440mg	トリスヒドロキシメチルアミノメタン	2,420mg
KCl	620mg	ウシ血清アルブミン	400mg
$CaCl_2$	20mg	プロマイン D	500mg
$MgCl_2$	8mg	ウシ血清アルブミン・プロマイン D 添加前にクエン酸で pH を調整（pH 7.25）	
$NaHCO_3$	20mg		

いずれも溶液 100ml 中の重量

Ocean fish in general

[sodium citrate solution[20]]

sodium citrate (dihydrate)

black tuna

[glucose solution[21]]

glucose

grey mullet

[Ringer's solution for marine fish[22]]

carp

[Ringer's solution for fresh-water fish[23]]

[artificial seminal plasma for carp[23]]

salmon in general

[Mounib's diluting solution[5]]

sucrose

glutathione (reducing type)

[Stein's diluting solution (V2)[6]]

glucose

glycine

(to 100 ml of this solution 20 g of chicken egg yolk is added)

[Stoss's diluting solution[24]]

trishydroxymethyl-aminomethane

bovine serum albumin

promine D

all weights are for 100 ml solutions

pH is adjusted with citric acid (pH 7.25) before adding bovine serum albumin & promine D

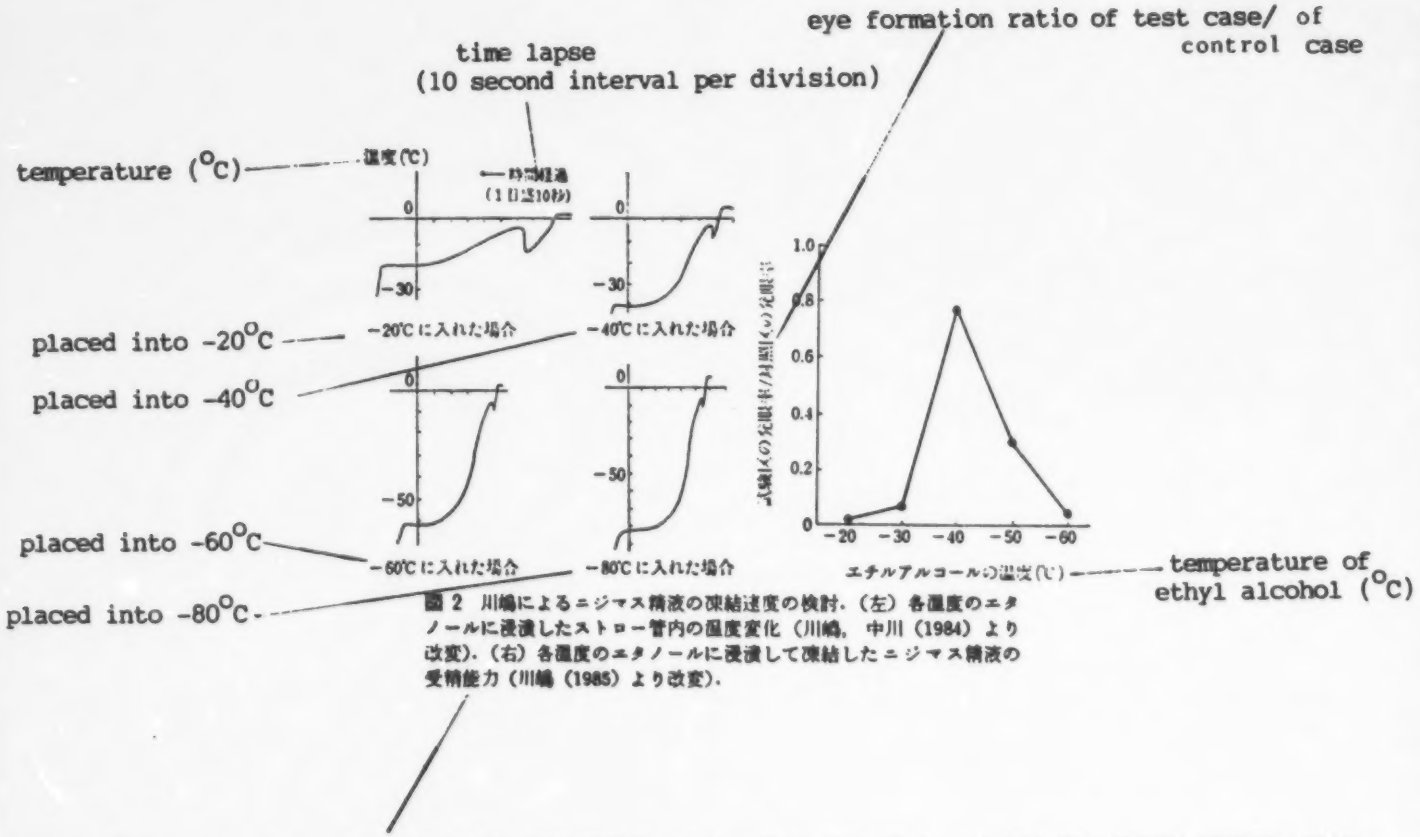

図 2 川嶋によるニジマス精液の凍結速度の検討．（左）各温度のエタノールに浸漬したストロー管内の温度変化（川嶋，中川（1984）より改変）．（右）各温度のエタノールに浸漬して凍結したニジマス精液の受精能力（川嶋（1985）より改変）．

Figure 2. Investigations by KAWASHIMA on the Freezing Rate of Rainbow Trout Spermatozoa.

(left) Temperature changes of the straws immersed in ethanol at various temperatures (adapted from KAWASHIMA, NAKAGAWA (1984)).

(right) Fertilizing Capabilities of rainbow trout sperm which had been frozen by immersing in ethanol at various temperatures (adapted from KAWASHIMA (1985)).

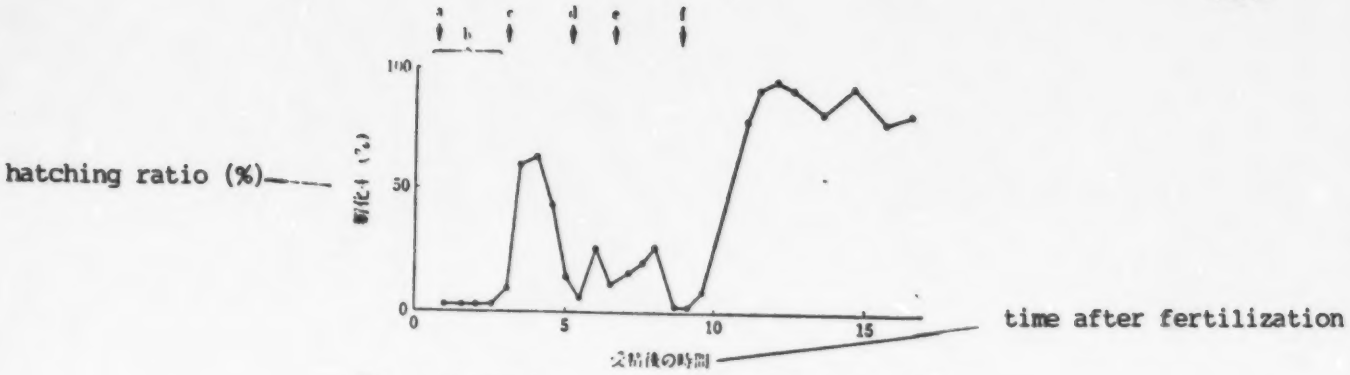

図 3 各発生段階のキュウセン卵に 30 分間低温刺激を与えた場合の孵化率．卵はあらかじめ基準水温 (27℃) に設定した海水中に収容し，一定時間ごとに発生段階を確認してから，4℃ の海水中に入れ，30 分間静置した後に再び基準水温に戻した．a：2 細胞期，b：卵割期，c：初期胞胚期，d：初期嚢胚期，e：胚体出現期，f：原口閉鎖期 (佐々木，黒倉，未発表)．

(Halichoeres poecilopterus (Temminck & Schlegel)

Figure 3. Hatching Ratios for Kyusen Eggs at Various Stages of Development when Subjected to Low Temperatures. The eggs were stored beforehand in sea water set at a standard temperature (27°C), then at selected time intervals the stages of development were checked and then transferred to 4°C sea water and allowed to stand for 30 minutes before bringing back to standard temperature. a: 2-cell stage, b: cleavage stage, c: early blastula stage, d: early gastrula stage, e: embryo-forming stage, f: blastopore closing stage (SASAKI, KUROKURA, unpublished results).

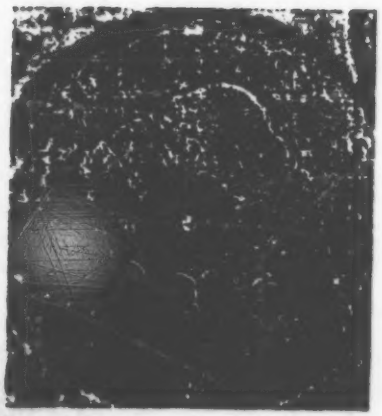

図 4 卵割期に低温刺激 (2℃, 1時間) を与えたマダイ卵．基準水温 17℃．

Figure 4. Sea bream egg which was subjected to low temperature (2°C, 1hour) during its cleavage stage. Standard water temperature is 17°C.

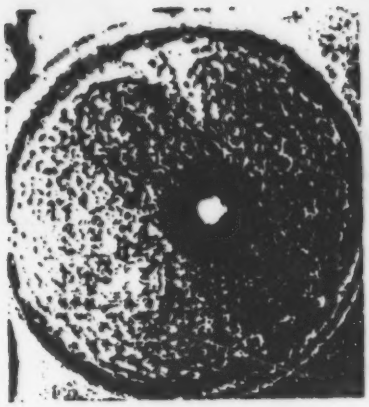

図 5 初期囊胚期に低温刺激を与えたマダイ卵.

Figure 5. Sea Bream Egg subjected to low temperature during the early gastrula stage.

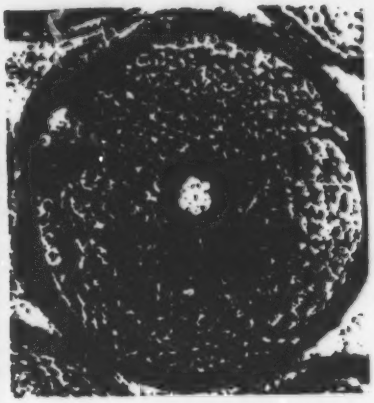

図 6 初期囊胚期に低温刺激を与えたマダイ卵.

Figure 6. Sea Bream Egg subjected to low temperature during the early gastrula stage.

Table 2. The Tolerance to Freezing at Various Stages of Development for Fertilized Flounder Eggs (OKAMOTO, KUROKURA, unpublished results).

表 2 ヒラメ受精卵の各発生段階における凍結耐性（岡本，黒倉，未発表）

発生段階	対照区*		実験区**	
	孵化率 (%)	奇形率 (%)***	孵化率 (%)	奇形率 (%)***
64 細胞期	80	6	0	—
桑実胚期	78	0	0	—
胞胚期	80	0	0	—
囊胚期	100	5	0	—
胚体出現期	100	10	0	—
眼胞形成期	95	11	0	—
耳胞形成期	100	5	67	100
レンズ形成期	100	5	62	100
尾鰭伸長期	100	5	57	92

* 各発生段階で，DMSO (2M) を含む海水中に 20 分間浸漬後，再び海水中で発生を続けた卵．
** DMSO を含む海水に浸漬後，−5℃/分で −20℃ まで冷却，その後，室温で発生を進めた卵．
*** 全孵化仔魚に対する奇形魚の百分率．

Development Stage
Control Section*
Experimental Section**

64 cell stage
morula stage
blastula stage
gastrula stage
embryo-forming stage
eye vesicle forming stage
ear vesicle formaing stage
lens forming stage
pinna caudalis growth stage

hatched ratio (%)

deformed ratio (%)***

*For the various stages of development, the eggs were soaked for 20 minutes in DMSO (2M) containing sea water, and development was continued in sea water.

**After soaking in DMSO containing sea water, the eggs were cooled to -20°C at -5°C/minutes, and then developed at room temperature.

***Percentage of deformed fish relative to the whole hatched fingerlings.

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CHAPTER 17: BREEDING BASED ON THE CHROMOSOME ENGINEERING METHOD

Hiroshi ONOZATO

1. INTRODUCTION

Fishes and shellfishes are an important source of protein for the Japanese. Since the establishment of the 200 nautical mile limit imposed for off-shore fishing", situations regarding deep-sea fishing have become severe and this has made the conversion from the fishing industry of the past involved in catching to the fishing industry involved in cultivation inevitable. As a result of the astounding technological progress made in the cultivation of seafood in Japan during past 20 to 30 years, the amount of production has grown and it has reached a stage of holding an important position in the coastal fisheries. However, there is a major difference when the fisheries industry is compared to agricultural and

" Translator's note: an assumption made on the exact wording in the original text "since entering the age of 200 nautical miles".

Canada

livestock industries that have a similar purpose of raising organisms. This difference lies on the point that in other industries breeding has been practiced for a several thousands of years, consequently, the improvement in the varieties available today has progressed to an extent that could not have been imagined from the original wild stock. On the other hand, in fisheries, with the exception of a very limited number of varieties, such as goldfish and colored carp, the reality is that most of the varieties that typify the cultivation such as young yellowtail, eels, scallops and laminaria are cultivated unchanged from their wild varieties. It goes without saying that breeding has a great economical effect in many ways such as improvement in both yield and quality. For this reason, there is no doubt that in fisheries, too, the productivity of cultivation will be greatly improved by the introduction of breeding. The breeding was done in the past by repeating the selection and crossing processes. However, this method requires many years. Since breeding is about to be used in the fisheries, an effort must be made in the active utilization of new methods, in addition to the conventional breeding methods, in order to obtain effective breeding results in a short period of time.

Fishes and shellfishes in general are characterized by external fertilization and by the fact that the number of eggs laid by an individual fish is in a higher order of magnitude than those discharged by the mammals. This fact is very

favorable for the manipulation of the eggs by various means during the time when they start to develop. Much research concerned with chromosome manipulation in fishes has been done in the last ten years because of this advantage, and as a result ample knowledge is being accumulated.

2. OUTLINE OF CHROMOSOME MANIPULATION

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An ovum by undergoing the maturation division twice successively during the process of maturation reduces the number of chromosomes, which have previously been multiplied to four sets by duplication, from four sets to one set. In other words, although homologous chromosomes become divalent chromosomes by pairing, each chromosome has already undergone duplication to become tetrad chromosomes. With the first maturation division, the homologous chromosomes are separated and one half is discarded to the exterior of the egg to form the first polar body. With the second maturation division, each chromosome that has remained in the egg aligns itself 237
along the duplication section with the half of the chromosomes being discarded to the egg exterior to form the second polar body. On the other hand, the chromosomes that have remained in the egg form the female pronucleus. The spermatozoon that has entered the ovum by fertilization swells to form the male pronucleus, and both pronuclei become fused by adhesion and come to possess diploid chromosomes. At what stage of maturation division the fertilization takes place is dependent on the species. In the case of fishes, the division is

suspended in the middle of the second maturation division and ovulation takes place. The entrance of the spermatozoon into the ovum becomes the stimulant for the restart of the second maturation division. On the other hand, with mollusk, the fist maturation division is completed after fertilization. The number of chromosomes, of which four sets were present before the first maturation division, is reduced to two sets by it, then to one set by the second maturation division, and is subsequently restored to two sets by the fertilization (Figure 1 (a)).

When a spermatozoon, which had been rendered genetically inactive by a treatment with radioactive rays or chemicals before fertilization, is used to fertilize a normal egg, the development is started only in the egg nucleus and a haploidic embryo having only one set of chromosomes is formed (Figure 1 (b)). Such development is called the gynogenesis. Since haploidy is a fatal condition the embryo dies around the time of hatching. In order to obtain an embryo which is capable of surviving, cell division must be suppressed to arrest the haplosis so that it can be made into a diploid. The cell division to be suppressed can be either one of the first maturation division (Figure 1 (c)), second maturation division (Figure 1 (d)), or the first egg cleavage (Figure 1 (e)). Although the egg cleavage taking place from the second cleavage on can be suppressed, the suppression of the first cleavage is the ideal since the synchronism of the cell division becomes

more disturbed with the successive cleavage. In fishes, since the first maturation division takes place inside the body its suppression is difficult with species other than those that are capable of external ovulation. On the other hand, with mollusk, since the first maturation division takes place after fertilization polyploidization is possible by any mean. When a normal spermatozoon is used for the fertilization instead of one which had been rendered genetically inactive and the first or the second maturation division is suppressed, a triploid having three sets of chromosomes (Figure 1 (f)) is formed since a female pronucleus having two sets of chromosomes is united with a male pronucleus having one set of chromosomes. On the other hand, if the development was allowed to proceed after the fertilization with a normal spermatozoon and if the first egg cleavage is suppressed, a tetraploid is formed (Figure 1 (g)). In these instances, if a heterogenic spermatozoon is used then an allotriploid or allo tetraploid is formed respectively. As opposed to gynogenesis, when the egg nucleus is rendered genetically inactive by such means as exposure to the γ-rays before fertilization, and is then fertilized with a normal spermatozoon, the development starts with the chromosomes of one set that originate only from the sperm nucleus to form an androgenetic haploid (Figure 1 (h)). In order to make it into a diploid that is capable of survival by means of polyploidization the first egg cleavage needs to be suppressed (Figure

1 (i)) since it is not possible to suppress the maturation division of the egg. A normal fertilized egg whose first and second maturation are suppressed becomes a penta ploid while the suppression of its second maturation division and first cleavage results in the formation of a hexa ploid.

3. HERTWIG EFFECT AND THE INDUCTION OF UNISEXUAL GENERATION

In order to determine an appropriate dose for inducing the gynogenesis or androgenesis, the gametes of one sex were exposed to various rays such as γ-rays, x-rays or ultraviolet rays ranging from a low dosage to high dosage, then fertilized with the normal gametes of the other sex, and the examination of the subsequent developmental processes revealed findings of great interest. The survival rate of fertilized egg for each dose obtained when spermatozoa of salmon were exposed to various doses of γ-rays ranging from 10^2 to 10^5 rad is given in Figure 2 (a) and Figure 2 (b). It was found that the survival rate was decreased as the dose of exposure was increased up to 10^2 to 10^4 rad, whereas once the dosage exceeded 10^4 rad the survival rate recovered as the dose was increased and at 10^5 rad about 90% were alive even after 40 days. Such apparently contradictory phenomenon that accompany the increase in dosage is known as the Hertwig effect[13]. This effect is being interpreted as follows: the exposure to the rays causes the genes to undergo mutation and the chromosomes to become severed, thus having a damaging effect on the development of the embryo resulting in a reduction in the survival rate; but

when the dose is increased further, the harmful chromosomes originating from the father are reduced in number for they fall off and the subsequent recovery takes place since the development takes place with normal chromosomes originating from the mother only[31]. Gynogenesis takes place when the chromosomes originating from the father disappear completely. In this manner the gynogenesis has been induced successfully using a high dose of γ-ray exposure in the 100,000 rad range with rainbow trout (S. gairdneri)[8,40], coho salmon (Oncorhynchus kisuch)[45], Salm trutta[39], Pleuronectes platessa[39] and Plactichthys flesus[39], carp (Cyprinus carpio)[26], and Oryzias latipes[15] or using the x-rays in sturgeon (Acipenser guldenstadii, A. ruthenus, and Huso huso)[46,47] and carp[6]. Because the γ-rays and x-rays have high penetration power they are suited for giving uniform exposure to a large amount of spermatozoa, but the use of these rays requires large-scale facilities and is also highly hazardous. Since the Hertwig effect also manifests itself when ultraviolet rays are used instead of these ionizing radiations, it is possible to induce gynogenesis (in Ctenopharyngodon idella[37], Brachydanio rerio[53], rainbow trout[24,37], Oncorhynchus masou[37], char Salvelinus leucomaenis[37], Osmerus aperlanus mordax[37], Oryzias latipes[16,27]. and carp[62]). It is known that similar gynogenesis can be induced by a chemical substance, for

example, toluidine blue (in Oryzias latipes[66]), dimethyl sulphate (in rainbow trout[61]) are some that can be listed.

Although only very little research has been done on the subject of androgenesis, in which the development is started by the sperm nucleus as the base, in comparison to the research concerning the gynogenesis, successful attempts have been made in some species of fish. Androgenetic haploids have been successfully obtained by Romashov and Beyaeva[46] in loach (Misgurunus fossilis), by Purdom[39] in Pleuronectes platessa and Platichtys flesus, by Arai et al.[4] in Oncorhynchus masou and by Parson and Thorgaard[38] in rainbow trout. Androgenesis has a lower rate of incidence than the gynogenesis for the reason that the exposure to the γ-rays not only destroy the egg nucleus but also affects DNA, RNA and protein of the cytoplasm.

4. NATURAL UNISEXUAL GENERATION

In the realm of nature, besides some species of fishes that reproduce by the gynogenesis, unisexual generation also takes place in some instances as a result of hybridization or overmaturation of the ovum. The fishes that reproduce by means of a natural unisexual generation are known as the parthenogenetic fish (all female fish) and the genera of Poecilia[14], Poeciliopsis[48] and Carassius[18] are known to belong to this category. Haplosis is prevented in these fishes by eliminating the first maturation division and the union of the egg and sperm nuclei is prevented by suppressing the activation of the spermatozoon after its entry into the ovum.

For this reason, the resulting offspring is a genetic copy of the mother, forming a clone[17)433)].

Some known examples of crossings where unisexual generation is induced by hybridization are the insemination of gold fish eggs with the spermatozoa of Morone chrysops[52)], crossing of Ctenopharyngodon idella with carp[25)], coho salmon with brook trout (Salvelinus fontinalis)[30)467)], and Platichthys flesus with halibut (Hippoglossus hippoglossus)[42)], and gynogenetic diploids or haploids are formed, though at a low rate. In a similar manner, an example of an occurrence of androgenetic haploids as a result of the crossing of Ctenopharyngodon idella with carp has been reported[50)]. On the other hand, Yamazaki[74)] reported that the rate of incidence of androgenetic haploids in salmon increases with the number of days after ovulation. Although the incidence of natural unisexual generation is low in general, the incidence of gynogenetic diploids takes place at a relatively high frequency in the crossing of coho salmon with brook trout[29)].

5. Polyploidization OF CHROMOSOMES

The majority of the individuals obtained by artificial unisexual generation are haploids and the ratio of normal individuals produced by natural diplosis is generally below the level of a few %. An artificial treatment is required in order to increase the rate of polyploidization. The polyploidization treatment can also be utilized to form polyploids. The polyploidization takes place when the chromosomes, which have doubled in number by

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duplication prior to division as a result of the suppression of the cell division, are retained in the original cell in that state. Although chemical substances such as colchicine[49] and cytochalasin B[2),43)&44)] have also been used as a tool for the suppression of cell division, treatments using thermoshock or hydraulic pressure are more widely used. In the thermoshock treatment, the fertilized egg is immersed in -1.7 to 5°C water immediately after fertilization for 10 minutes. This method is being tested using the three-spined stickleback (Gasterosteus aculeatus)[54], carp[10),23),26)&29)], bitterling Rhodeus ocellatus ocellatus[64], Ictalurus punctatus[72], Pleuronectis platessa[41] as well as Platichthys flesus[41], brook trout[19], rainbow trout[7)&70)], tilapia[65] and others. However, in cold-water fishes such as salmonid fishes this method is not very effective, whereas in tilapia, which favors hot water, better results have been obtained at 11°C than at 4°C. With salmonid fishes a high-temperature treatment is more effective than the low-temperature treatment[7),60)&65)] and this is also being attempted using sturgeon[69] and three-spined stickleback[54]. On the other hand, the hydraulic pressure treatment also has an apparent effect on the polyploidization. Dasgupta[9] was the first to demonstrate the effectiveness of 241
the hydraulic pressure treatment on the polyploidization. Since then this treatment method has also been used to obtain high ratio polyploidization in fishes using Brachydanio rerio[53], salmonid fishes[24),32)&34)] and Ctenopharyngodon idella[5] (Grass Carp). The

disappearance of the spindle, which is the division device, as a result of treatment with hydraulic pressure has been confirmed cytologically (Figure 3). Consequently, it is thought that the spindle is destroyed by the polyploidization, thus making it impossible for the chromosomes to move.

6. GENETIC CHARACTERISTICS OF UNISEXUALLY GENERATED DIPLOIDS

6.1 Genetic Characteristics

Unisexual generation produces a few interesting genetic characteristics thus making the genetic purification and the control of the sex (of the offspring) possible. In the case 242
of gynogenesis, the significance of the polyploidization treatment varies depending on the stage of the maturation or development of the egg on which it is performed. When polyploidization is allowed to take place by suppressing the first maturation division, if no pairing takes place the maternal genes are passed on unchanged, thus the resulting offspring is a genetic copy of the mother (Figure 4 (d)). If pairing takes place, then there is a possibility that the heterozygote of the paired section becomes a homozygote in the offspring (Figure 4 (h)). In the case of the suppression of the second maturation division, a completely homogenic individual is obtained if no pairing takes place (Figure 4 (c)), and the heterozygote at the paired section is passed on to the offspring unchanged if pairing takes place (Figure 4 (g)). In the case of the suppression of the first egg cleavage, a completely homogenic individual is obtained regardless of whether pairing took place or not

(Figure 4 (b) & (f)). The individual whose division was not suppressed becomes a haploid (Figure 4 (a) & (e)). The gynogenetic diploid becomes a completely homogenic animal since it is formed by the suppression of the first egg cleavage.

6.2 Sexual Segregation

As in the case of mammals, in fishes, too, the sex is generally determined by the combination of the sex chromosomes[74]. Although many ways in which the sex is determined by the sex chromosomes are known, they can be roughly divided into the female homogenic and male homogenic types. The most general example of the female homogenic type is what is called the XY type. In this case, the individual formed by the gynogenesis always becomes a female regardless of the stage at which the polyploidization treatment is applied. On the other hand, with the female heterogenic ZW type, a female WW and a male ZZ are formed in situations demonstrated in Figure 4 (b), (c) and (f). Although ZZ is a normal male, WW is called a superfemale and does not exist in nature. When this 243
superfemale is crossed with a normal male (ZZ), all of the offsprings produced will be ZW females. On the other hand, in situations demonstrated in Figure 4 (d) and (g), females are produced, whereas in the case of (h) a superfemale is produced in addition to a normal male and female. Although it is not known whether pairing takes place between sex chromosomes, in the case of fishes, the fact that in many species X chromosomes cannot be distinguished morphologically from Y chromosomes, or

Z chromosomes from W chromosomes, gives enough cause to consider that the pairing of both sex chromosomes is possible. Moreover, just as YY is fatal in mammals, it is possible that WW is fatal. If this is true, then only males are produced in situations demonstrated in Figure 4 (b), (c) and (f), however, since no case of gynogenesis of the ZW type has been reported this is not known.

The androgenetic diploid formed as a result of the suppression of the first egg cleavage is completely homogenic. Consequently, if the sex determination is according to the XY type then XX and YY are isolated in the ratio of 1:1. In contrast to WW, YY is called a supermale and all offsprings produced by the crossing of this individual with a normal female are males only. If the supermale is fatal then only females are produced. On the other hand, in the case of ZW, only ZZ males are produced.

7. CHARACTERISTICS OF THE POLYPLOIDS

7.1 Genetic Characteristics

Since the triploid is obtained by fertilizing normal spermatozoon and by suppressing the maturation division, its genotype is the that of the gynogenetic diploid (Figure 4 (c), (d), (g) and (h)) with the gene of the spermatozoa added to it. Consequently, sometimes situations arise where the allele mass ratio is 2:1 (AAB etc.) or where three alleles are present (ABC).

In the case of hybrids, genetic effects that are different from those found in the diploidic hybrid can be expected. On the other hand, with the tetra ploid, only the quantitative doubling of each gene takes place and the allele mass ratio remains unchanged.

7.2 Sexual Segregation

In the animals of the XY type, the triploid sex chromosomes are either XXX or XXY, whereas in the animals of the ZW type three forms ZZZ, ZWW or ZZW can appear if pairing takes place. It has been revealed that XXX produces the ovaries whereas XXY produces the testes. Consequently, the presence of a gene that induces the male in the Y chromosome is considered. Although the mechanism of sex determination in rose bitterling
Rhodeus ocellatus ocellatus is uncertain, all triploids become males[64].

7.3 The Size of Nuclei and of Cells

It is generally known that as the ploidy is increased the nucleus and the cell become large. Figure 5 was obtained 244
when the minor axis of the embryonic brain cell of rainbow trout was determined and its cube for each individual was plotted. When the average for the diploid (normal development) is assumed to be 2, then the relative volumes of the haploid (gynogenesis), triploid (pressurization treatment 15 minutes after the fertilization) and tetra ploid (pressurization treatment at 5 and 5 1/2 hours after fertilization) are in the vicinity of 1, 3, and 4, respectively, demonstrating that the

ploidy is proportional to the nuclear volume. In a similar manner, the cellular size is also nearly proportional to the ploidy. However, since the sizes of the organs remain unchanged, a phenomenon in which the number of cells that constitute the organs is decreased is observed (Figure 6). The erythrocyte size is often used to determine the ploidy[1),68)&71)].

7.4 Fertility

It is a well known fact that the triploid becomes sterile, as seen in seedless watermelons. In fishes, too, the retardation in the gonad development is observed in most cases[10),21),63)&73)] and the extent of retardation is greater in females than in males[22),40)&55)]. In rainbow trout[39)] and Plaice[20)], the spermatozoal development was observed in the triploid males. Although the spermatozoa of the latter were fertile, all subjects died during the embryo developmental stage. On the other hand, no spermatozoa were formed in bullhead[73)] and Oncorhynchus masou[35)]. Although it is not sufficiently understood why the triploid becomes infertile, it is thought that the failure in pairing interferes with the subsequent gamete formation. Although females enter the mating season at a very young stage, because males enter it at a considerably later stage, it is thought that the testes are developed to some degree as a result of the spermatogonic division that repeatedly takes place during this period of

delay. For the tetra ploid, no particular cause for infertility comes to mind. 246

7.5 Survival Ratio of Fatal Hybrids

The most interesting phenomenon associated with the triploid is the known case that fatal hybrids can be changed into one capable of survival. Although the crossing of salmon ♀ x char ♂ is fatal, it can be changed into one capable of survival by transforming it to a triploid[3]. The cause for this is as yet totally unclear.

8. THE APPLICATION OF CHROMOSOME MANIPULATION

8.1 The Control of the Sex

Since in many of the species of aquatic organisms their commercial value or the efficiency of their cultivation is dependent on whether they are male or female, the artificial control of the sex is important and chromosome engineering is an extremely useful means of achieving this. In animals whose sex is determined by the XY type, only females are produced by the male-female generation. However, the gynogenesis does not progress well and often results in the manifestation of inbred debility. For this reason, in order to produce all females more readily, attempts have been made to obtain all female offsprings by performing a sex conversion of gynogenetic individual to an XX male and then crossing this with a normal female and this has been put into practice partially. The advantages of this method can be listed as follows: female-only seeds and saplings can be obtained by means of simple

fertilization; if a small number of males can be obtained then they can be used to fertilize a large number of eggs thus making the production of a large number of females possible; and the interference by inbred debility can be eliminated. An outline by Yamazaki[74] exists on the topic of sex conversion. On the other hand, YY that can be obtained by androgenesis can be used for the production of all male fish. It was confirmed by Onozato[36] that the male obtained by the androgenesis is a supermale (YY).

8.2 Sterilization

The sterilization of the triploid was already explained. Although it can be construed that the energy that is normally used for the formation of gonads will be used for body growth as a result of sterilization and that growth will be enhanced by that much, however, only in a few cases was the growth noticeably facilitated by the formation of triploids. In spite of this, depending on the purpose of the cultivation, the sterilization caused by the triploidization becomes an extremely effective means.

a) The longevity effect and increased size

Examples of animals that spawn only once in a lifetime and die afterwards are not few among aquatic animals. The maximum lifespan as well as maximum body length of these fish species are stipulated by maturation. It is possible to expect that the inherent lifespan and the maximum body length can be

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manifested by sterilization and this will result in marked prolongation of the lifespan as well as increase in size.

b) The prevention of depreciation in the commercial value accompanying maturation

A significant decline in the commercial value of 247
Pacific salmon is observed as they reach maturity. Some decline, whether large or small, in commercial value is also observed with other varieties of salmon and trout, in addition to the fact that they cannot be shipped for sale during spawning season. Sterilization is effective in eliminating such disadvantages.

c) The prevention of natural decline accompanying maturation

Although they do not all die as is the case with Pacific salmon, other species of salmonid fishes are generally more prone to succumbing to aquatic bacteria after spawning and thus decrease greatly in number. Sterilization has a bearing on the prevention of such loss.

d) The prevention of growth retardation during spawning season

In more than a few species of fishes, the growth is not only retarded due to repeated fasting but their weight is also decreased significantly during spawning season. Because tilapia spawn frequently, the growth is significantly checked in those individuals that have reached the age of maturity, the females in particular. It is expected that sterilization will be effective in preventing the retardation of growth.

e) The prevention of overcrowding of the population

In the United States, Grass Carp Ctenopharyngodon idella are being released with the aim of preventing the overgrowth of aquatic plants in lakes and marshes. However, serious environmental destruction was brought about by the over proliferation of these released fish for they consumed all of the aquatic plants. The release of sterilized fish will prevent such secondary pollution. Tilapia are overly reproductive and tend to become overcrowded. The creation of sterilized fish is being greatly sought by those who are involved in their cultivation.

8.3 Genetic Fixation and Clone Preparation

In the unisexually generated individual formed by the suppression of the first egg cleavage, all genes are homozygotes since one set of chromosomes have been doubled to two sets. With the completely homogenic animal produced in such a manner, the genetic isolation as a result of meiosis cannot take place during the time of gamete formation, thus genetically uniform gamete is produced. When the offspring is again produced by the monogenesis in the following generation, the offspring becomes a genetic copy of the mother and the siblings become genetically uniform clones. Clones are ideal animals to be used for experiments, and, at the same time, superior characters found in the parental generation can be passed on unchanged to the next generation. This is what is called genetic fixation. When sex conversion is performed in a

part of a clone then the clone can be maintained by intra-clone breeding, and inter-clone breeding becomes possible thus opening a way for the application to attain heterosis and clone improvement. Such concepts are the basis for the clone production that are now in progress using Brachydanio rerio[53] (top minnow) and Oryzias latipes[27].

8.4 The Preparation of Gene Maps

In the male-female generated diploid and triploid produced by the suppression of the second maturation division, the heterozygote of the mother is passed on to the offspring unchanged only when mating takes place (Figure 4 (g)). Consequently, the rate at which the heterozygote of the mother is passed on to the offspring represents the crossing-over rate of that gene and the crossing-over rate reflects the distance from the centromere to the locus. Therefore, the relative distance of the centromere and locus can be obtained from the crossing-over rate. The distance of the locus from the centromere has been obtained for some loci in rainbow trout[12)&58], Macropodus opercularis[11], ayu[57], flounder[56] and other species of fish. It has been suggested that in general the crossing-over rate in fishes is extremely high, a numerous loci are present near the tip of the chromosome and that double crossing over does not take place.

8.5 Preserving the Gene Stocks and the Species on the Brink of Extinction

Careless development that is in progress in various

areas of Japan in recent years has brought about a disastrous blow to the habitat of freshwater as well as saltwater fish, and some fish species are endangered. Such situations must be curtailed not only for the sake of dealing with a sensational problem of the extinction of those species but also from the perspective of dealing with the loss of valuable genetic resources. Although the preservation of seeds has been established as a method of preserving the genetic resources of 249
plants, in animals there has not been any means available to achieve this other than raising individual animals. Androgenesis is thought to be an effective tool as a method of preserving the genetic resources or seeds. In other words, by preserving these seeds in a form of frozen spermatozoa, it is possible to develop them into fully grown individuals by means of foster ova of closely related seeds. On the basis of a such concept, the author has confirmed the survival capacity and fertility of the androgenetic diploid, and demonstrated that androgenesis can be induced using spermatozoa which had been kept frozen and that the androgenetic diploid can be produced in a foster heterologous egg[36]. A red-spotted masu trout produced in a foster Oncorhynchus masou egg is given in Figure 7.

9. CONCLUSION

Chromosome engineering in fishes has made remarkable progress in the last 20 years, and the application of this method in various fields is likely to take place in the near

future. Moreover, a method of polyploidization by the fused spermatozoon was recently introduced by Ueda et al.[63] of the Utsunomiya University. This method is based on a concept that is entirely different from that of the gene manipulation method described up to this point, and it can be said to be an epoch-making method. Concerning the existing gene manipulation method, the suppression of the first egg cleavage has been most difficult and has posed many problems of a technical nature that are yet to be resolved, however, this polyploidization as a result of fertilization by fused spermatozoon can replace the first egg cleavage. It is expected that this method will further aid in simplifying the production of tetraploids and androgenetic diploids. As well, there is a need to develop in future a technology to enable the addition of only specific chromosomes or the addition of a specific portion of a chromosome. If this were to be realized, then the potential of chromosome engineering will be enlarged a step further.

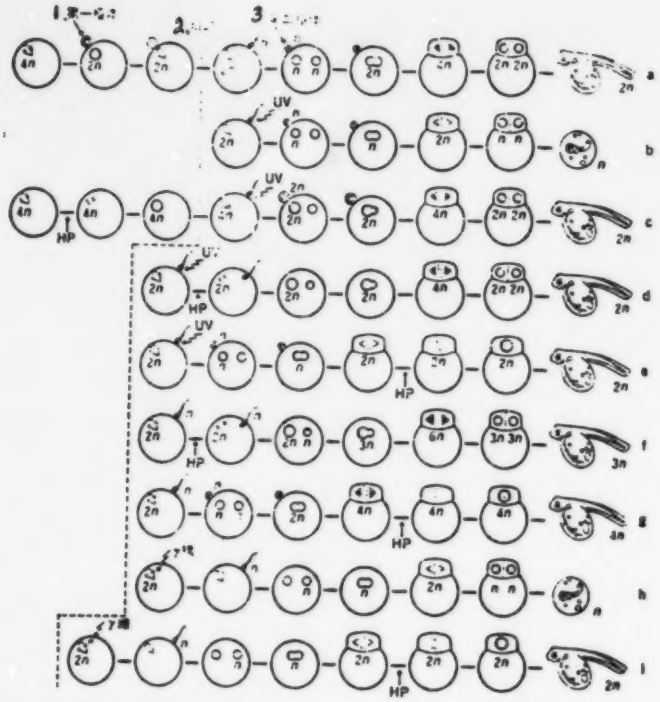

図1 染色体操作[31]. (a) 正常発生, (b) 雄性発生半数体, (c) 第1成熟分裂の阻害による雄雌発生2倍体, (d) 第2成熟分裂の阻害による雌性発生2倍体, (e) 第一卵割の阻害による雌性発生2倍体, (f) 3倍体, (g) 4倍体, (h) 雄性発生半数体, (i) 雄性発生2倍体. HP: 加圧(分裂阻害), UV: 紫外線

Figure 1: Chromosome manipulation[31].
(a) normal generation
(b) gynogenetic haploid
(c) male-female generated diploid formed by the suppression of the first maturation division
(d) gynogenetic diploid formed by the suppression of the second maturation division
(e) gynogenetic diploid formed by the suppression of the first egg cleavage
(f) triploid
(g) tetra ploid
(h) androgenetic haploid
(i) androgenetic diploid
HP: pressurization (the suppression of the division)
UV: ultraviolet rays
1. the first polar body
2. ovulation
3. the second polar body

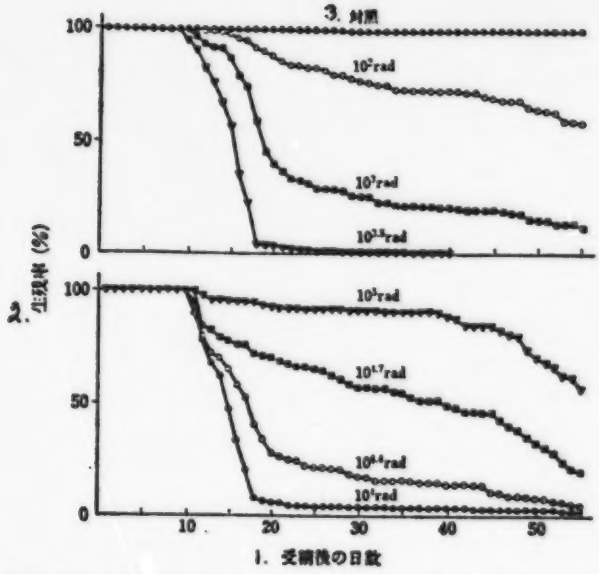

図 2 種々の線量の線をサケの精子に照射したときの受精卵の生残率.
(a) 低線量域, (b) 高線量域

Figure 2: Survival rate of fertilized egg when salmon spermatozoa are exposed to various doses of rays.
(a) low dosage area
(b) high dosage area
1. number of days after fertilization
2. survival rate (%)
3. control

図 3 加圧処理前後のサケの胞胚期細胞. (a), (b) 加圧処理前, (c), (d) 加圧処理直後. (a), (c) 有糸分裂中期像, (b), (d) 有糸分裂後期像

Figure 3: Salmon cell of the blastula period before and after the pressure treatment.
(a) & (b) before the pressure treatment
(c) & (d) immediately after the pressure treatment
(a) & (c) image during the middle stage of the mitotic period
(b) & (d) image from the latter stage of the mitotic period

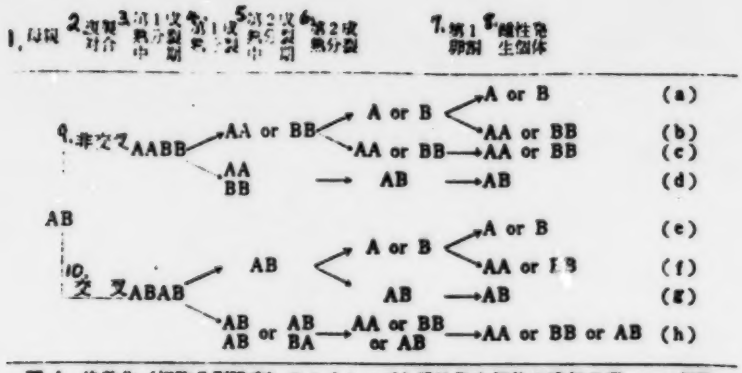

図4 倍数化（細胞分裂阻害）のタイミングと雌性発生個体の遺伝子型．⟶細胞分裂，⟶細胞分裂阻害

Figure 4: The timing of polyploidization (the suppression of the cell division) and the genotype of the gynogenetic specimen.
⟶ cell division -----> cell division suppressed

1. mother
2. duplication/pairing
3. middle of the first maturation division stage
4. the first maturation division
5. middle of the second maturation division stage
6. the second maturation division
7. the first egg cleavage
8. gynogenetic specimen
9. no pairing
10. pairing

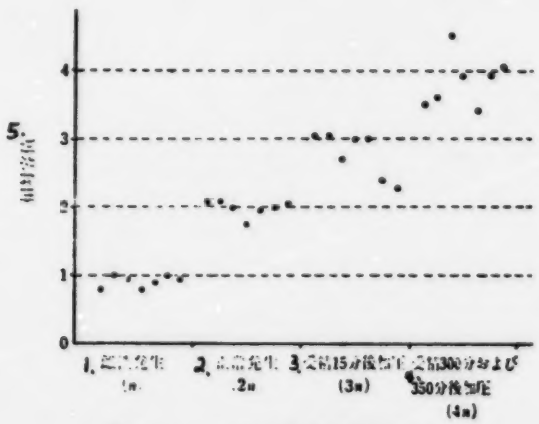

図5 ニジマス胚の脳細胞核の相対容積．

Figure 5: The relative capacity of the embryonic brain cell nuclei of rainbow trout.

1. gynogenesis (n)
2. normal generation (2n)
3. pressure applied 15 minutes after fertilization (3n)
4. pressure applied 300 minutes and 350 minutes after fertilization (4n)
5. relative capacity

図 6 ニジマスの発眼胚の倍数化に伴う組織変化. (a)~(d) 尿細管, (e)~(h) 消化管, (a)~(e) 半数体, (b), (f) 2倍体, (c), (g) 3倍体, (d), (h) 4倍体

Figure 6: Changes in the cells accompanying the polyploidization of the rainbow embryo at the stage of eye development.

(a) - (d) ureter
(e) - (h) digestive tract
(a) - (e) haploid
(b) & (f) diploid
(c) & (g) triploid
(d) & (h) tetra ploid

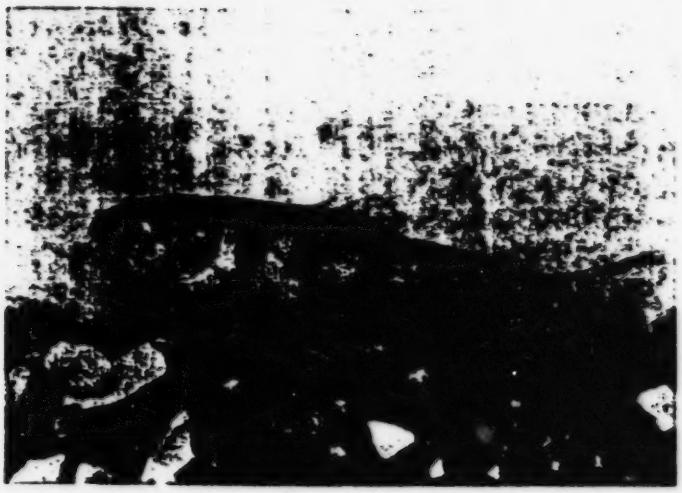

図7 サクラマスの卵を借りて作られた雄性発生アマゴ

Figure 7: Androgenetic red-spot masu trout produced by a foster egg of sockeye salmon.

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